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(21) International Application Number: PCT/CA97/00051 (22) International Filing Date: 27 January 1997 (27.01.97) (30) Priority Data: <table border="0" style="width: 100%;"> <tr> <td style="width: 30%;">08/592,541</td> <td style="width: 40%;">26 January 1996 (26.01.96)</td> <td style="width: 30%;">US</td> </tr> <tr> <td>60/021,673</td> <td>5 July 1996 (05.07.96)</td> <td>US</td> </tr> <tr> <td>60/021,700</td> <td>12 July 1996 (12.07.96)</td> <td>US</td> </tr> <tr> <td>60/029,895</td> <td>8 November 1996 (08.11.96)</td> <td>US</td> </tr> <tr> <td>60/034,590</td> <td>2 January 1997 (02.01.97)</td> <td>US</td> </tr> </table> (71) Applicants (for all designated States except US): HSC RESEARCH AND DEVELOPMENT LIMITED PARTNERSHIP [CA/CA]; Suite 5270, 555 University Avenue, Toronto, Ontario M5G 1X8 (CA). THE GOVERNING COUNCIL OF THE UNIVERSITY OF TORONTO [CA/CA]; 106 Simcoe Hall, 27 King's College Circle, Toronto, Ontario M5S 1A1 (CA). (72) Inventors; and (75) Inventors/Applicants (for US only): ST. GEORGE-HYSLOP, Peter, H. [CA/CA]; 210 Richview Avenue, Toronto, Ontario M5P 3G3 (CA). FRASER, Paul, E. [CA/CA]; 611 Windermere Avenue, Toronto, Ontario M6S 3L9 (CA). ROM-		08/592,541	26 January 1996 (26.01.96)	US	60/021,673	5 July 1996 (05.07.96)	US	60/021,700	12 July 1996 (12.07.96)	US	60/029,895	8 November 1996 (08.11.96)	US	60/034,590	2 January 1997 (02.01.97)	US	MENS, Johanna, M. [CA/CA]; 105 McCaul Street, Toronto, Ontario M5T 2X4 (CA). (74) Agent: RAE, Patricia, A.; Sim & McBurney, 6th floor, 330 University Avenue, Toronto, Ontario M5G 1R7 (CA). (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
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(54) Title: NUCLEIC ACIDS AND PROTEINS RELATED TO ALZHEIMER'S DISEASE, AND USES THEREFOR																	
(57) Abstract <p>The present invention describes the identification, isolation, sequencing and characterization of several human genes which interact with the presenilins, mutations in which may lead to Familial Alzheimer's Disease. These presenilin-interacting protein genes may be involved in the pathways which, when affected by mutant presenilins, lead to the development of Alzheimer's Disease. In addition, mutations in the presenilin-interacting protein genes, even in the absence of defects in the presenilins, may be causative of Alzheimer's Disease. Nucleic acids and proteins comprising or derived from the presenilin-interacting proteins are useful in screening and diagnosing Alzheimer's Disease, in identifying and developing therapeutics for treatment of Alzheimer's Disease, and in producing cell lines and transgenic animals useful as models of Alzheimer's Disease.</p>																	

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**NUCLEIC ACIDS AND PROTEINS
RELATED TO ALZHEIMER'S DISEASE,
AND USES THEREFOR**

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Field of the Invention

The present invention relates generally to the field of neurological and physiological dysfunctions associated with Alzheimer's Disease. More particularly, the invention is concerned with the identification, isolation and cloning of genes which are associated with Alzheimer's Disease, as well as their corresponding transcripts and protein products. The present invention also relates to methods for detecting and diagnosing carriers of normal and mutant alleles of these genes, to methods for detecting and diagnosing Alzheimer's Disease, to methods of identifying other genes and proteins related to, or interacting with, the genes and proteins of the invention, to methods of screening for potential therapeutics for Alzheimer's Disease, to methods of treatment for Alzheimer's Disease, and to cell lines and animal models useful in screening for and evaluating potentially useful therapies for Alzheimer's Disease.

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Background of the Invention

Alzheimer's Disease (AD) is a degenerative disorder of the human central nervous system characterized by progressive memory impairment and cognitive and intellectual decline during mid to late adult life (Katzman, 1986). The disease is accompanied by a constellation of neuro-pathologic features principal amongst which are the presence of extracellular amyloid or senile plaques, and neurofibrillary tangles in neurons. The etiology of this disease is complex, although in some families it appears to be inherited as an autosomal dominant trait. Linkage studies have identified three genes associated with the development of AD: β -amyloid precursor protein (APP) (Chartier-Harlin et al., 1991; Goate et al., 1991; Murrell et al., 1991; Karlinsky et al., 1992; Mullan et al., 1992), presenilin-1 (PS-1) (Sherrington, 1995), and presenilin-2 (PS-2) (Rogaev, 1995, and Levy-Lahad, 1995).

The presenilins are multi-spanning membrane proteins which were described in substantial detail in PCT Publication WO96/34099, the entire disclosure of which is incorporated herein by reference. Although the functions of the presenilins are unknown, a number of autosomal dominant presenilin mutations have been identified

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which are strongly associated with the development of early-onset, aggressive, Familial Alzheimer's Disease (FAD).

The present disclosure describes the identification, isolation, sequencing and characterization of several human genes which interact with the presenilins, mutations
5 in which may lead to FAD. These presenilin-interacting protein genes may be involved in the pathways which, when affected by mutant presenilins, lead to the development of Alzheimer's Disease. In addition, mutations in the presenilin-interacting protein genes, even in the absence of defects in the presenilins, may be causative of Alzheimer's Disease.

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Summary of the Invention

The present invention is based, in part, upon the identification, isolation, sequencing and characterization of several human genes, referred to herein as "presenilin-interacting protein genes" or "PS-interacting protein genes." The products of these genes are believed to interact in vivo with the human presenilin-1 proteins
15 and, therefore, are implicated in the biochemical pathways which are affected in Alzheimer's Disease. Each of these genes, therefore, presents a new therapeutic target for the treatment of Alzheimer's Disease. In addition, PS-interacting protein nucleic acids, PS-interacting proteins and peptides, antibodies to the PS-interacting proteins, cells transformed with PS-interacting protein nucleic acids, and transgenic animals
20 altered with PS-interacting protein nucleic acids, all possess various utilities, as described herein, for the diagnosis, therapy and continued investigation of Alzheimer's Disease and related disorders.

Thus, it is one object of the invention to provide isolated nucleic acids encoding at least a PS-interacting domain of a PS-interacting protein. These PS-
25 interacting proteins include mammalian S5a subunits of the 26S proteasome, the GT24 protein, the p0071 protein, the Rab11 protein, the retinoid X receptor- β , the cytoplasmic chaperonin, and several sequences identified herein as clones Y2H35, Y2H171, and Y2H41. Preferred nucleotide and amino acid sequences are provided herein. It is another object of the invention to provide probes and primers for these

PS-interacting protein genes, and to provide nucleic acids which encode small antigenic determinants of these genes. Therefore, preferred embodiments include sequences of at least 10, 15 or 20 consecutive nucleotides selected from the disclosed sequences.

5 Using the nucleic acid sequences and antibodies disclosed and enabled herein, methods for identifying allelic variants or heterospecific homologues of a human PS-interacting protein and gene are provided. The methods may be practiced using nucleic acid hybridization or amplification techniques, immunochemical techniques, or any other technique known in the art. The allelic variants may include
10 other normal human alleles as well as mutant alleles of the PS-interacting protein genes which may be causative of Alzheimer's Disease. The heterospecific homologues may be from other mammalian species, such as mice, rats, dogs, cats or non-human primates, or may be from invertebrate species, such as Drosophila or C. elegans. Thus, it is another object of the invention to provide nucleic acids which
15 encode allelic or heterospecific variants of the disclosed sequences, as well as the allelic or heterospecific proteins encoded by them.

 The it another object of the invention to provide vectors, and particularly expression vectors, which include any of the above-described nucleic acids. It is a further object of the invention to provide vectors in which PS-interacting protein
20 nucleic acid sequences are operably joined to exogenous regulatory regions to produce altered patterns of expression, or to exogenous coding regions to produce fusion proteins. Conversely, it is another object to provide nucleic acids in which PS-interacting protein regulatory regions are operably joined to exogenous coding regions, including standard marker genes, to produce constructs in which the
25 regulation of PS-interacting protein genes may be studied and used in assays for therapeutics.

 It is another object of the invention to provide host cells and transgenic animals which have been transformed with any of the above-described nucleic acids

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of the invention. The host cells may be prokaryotic or eukaryotic cells and, in particular, may be gametes, zygotes, fetal cells, or stem cells useful in producing transgenic animal models.

In particularly preferred embodiments, the present invention provides a
5 non-human animal model for Alzheimer's Disease, in which the genome of the animal, or an ancestor thereof, has been modified by at least one recombinant construct which has introduced one of the following modifications: (1) insertion of nucleotide sequences encoding at least a functional domain of a heterospecific normal PS-interacting protein, (2) insertion of nucleotide sequences encoding at least a
10 functional domain of a heterospecific mutant PS-interacting protein, (3) insertion of nucleotide sequences encoding at least a functional domain of a conspecific homologue of a heterospecific mutant PS-interacting protein, and (4) inactivation of an endogenous PS-interacting protein gene. Preferred transgenic animal models are rats, mice, hamsters, guinea pigs, rabbits, dogs, cats, goats, sheep, pigs, and non-
15 human primates, but invertebrates are also contemplated for certain utilities.

It is another object of the invention to provide methods for producing at least a functional domain of a PS-interacting protein using the nucleic acids of the invention. In addition, the present invention also provides substantially pure preparations of such proteins, including short peptide sequences for used as
20 immunogens. Thus, the invention provides peptides comprising at least 10 or 15 consecutive amino acid residues from the disclosed and otherwise enabled sequences. The invention further provides substantially pure preparations of peptides which comprise at least a PS-interacting domain of a PS-interacting protein, as well as substantially pure preparations of the entire proteins.

25 Using the substantially pure peptides and proteins enabled herein, the invention also provides methods for producing antibodies which selectively bind to a PS-interacting protein, as well as cell lines which produce these antibodies.

Another object of the present invention is to provide methods of identifying compounds which may have utility in the treatment of Alzheimer's Disease and related disorders. These methods include methods for identifying compounds which can modulate the expression of a PS-interacting protein gene, methods for identifying compounds which can selectively bind to a PS-interacting protein, and methods of identifying compounds which can modulate activity of a PS-interacting protein. These methods may be conducted in vitro or in vivo, and may employ the transformed cell lines and transgenic animal models of the invention. The methods also may be part of a clinical trial in which compounds identified by the methods of the invention are further tested in human subjects.

It is another object of the invention to provide methods of diagnosing or screening for inherited forms of Alzheimer's Disease by determining if a subject bears a mutant PS-interacting protein gene. Mutant PS-interacting genes may be detected by assays including direct nucleotide sequencing, probe specific hybridization, restriction enzyme digest and mapping, PCR mapping, ligase-mediated PCR detection, RNase protection, electrophoretic mobility shift detection, or chemical mismatch cleavage. Alternatively, mutant forms of a PS-interacting protein may be detected by assays including immunoassays, protease assays, or electrophoretic mobility assays.

It is also an object of the invention to provide pharmaceutical preparations which may be used in the treatment of Alzheimer's Disease and related disorders which result from aberration in biochemical pathways involving the PS-interacting proteins disclosed and enabled herein. Thus, the present invention also provides pharmaceutical preparations comprising a substantially pure PS-interacting protein, an expression vector operably encoding a PS-interacting protein, an expression vector operably encoding a PS-interacting protein antisense sequence, an antibody which selectively binds to a mutant PS-interacting protein, or an antigenic determinant of a mutant PS-interacting protein. These pharmaceutical preparations may be used to

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treat a patient bearing a mutant PS-interacting protein gene which is causative of Alzheimer's Disease or related disorders.

These and other objects of the present invention are described more fully in the following specification and appended claims.

5

Detailed Description of the Invention

I. Definitions

In order to facilitate review of the various embodiments of the invention, and an understanding of the various elements and constituents used in making and using the invention, the following definitions are provided for particular terms used in the description and appended claims:

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Presenilin. As used without further modification herein, the terms "presenilin" or "presenilins" mean the presenilin-1 (PS1) and/or the presenilin-2 (PS2) genes/proteins. In particular, the unmodified terms "presenilin" or "presenilins" refer to the mammalian PS1 and/or PS2 genes/proteins and, preferably, the human PS1 and/or PS2 genes/proteins as described and disclosed in PCT Publication WO96/34099.

15

Normal. As used herein with respect to genes, the term "normal" refers to a gene which encodes a normal protein. As used herein with respect to proteins, the term "normal" means a protein which performs its usual or normal physiological role and which is not associated with, or causative of, a pathogenic condition or state. Therefore, as used herein, the term "normal" is essentially synonymous with the usual meaning of the phrase "wild type." For any given gene, or corresponding protein, a multiplicity of normal allelic variants may exist, none of which is associated with the development of a pathogenic condition or state. Such normal allelic variants include, but are not limited to, variants in which one or more nucleotide substitutions do not result in a change in the encoded amino acid sequence.

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Mutant. As used herein with respect to genes, the term "mutant" refers to a gene which encodes a mutant protein. As used herein with respect to proteins, the term "mutant" means a protein which does not perform its usual or normal

physiological role and which is associated with, or causative of, a pathogenic condition or state. Therefore, as used herein, the term "mutant" is essentially synonymous with the terms "dysfunctional," "pathogenic," "disease-causing," and "deleterious." With respect to the presenilin and presenilin-interacting protein genes and proteins of the present invention, the term "mutant" refers to genes/proteins bearing one or more nucleotide/amino acid substitutions, insertions and/or deletions which typically lead to the development of the symptoms of Alzheimer's Disease and/or other relevant inheritable phenotypes (e.g. cerebral hemorrhage, mental retardation, schizophrenia, psychosis, and depression) when expressed in humans. This definition is understood to include the various mutations that naturally exist, including but not limited to those disclosed herein, as well as synthetic or recombinant mutations produced by human intervention. The term "mutant," as applied to these genes, is not intended to embrace sequence variants which, due to the degeneracy of the genetic code, encode proteins identical to the normal sequences disclosed or otherwise enabled herein; nor is it intended to embrace sequence variants which, although they encode different proteins, encode proteins which are functionally equivalent to normal proteins.

Substantially pure. As used herein with respect to proteins (including antibodies) or other preparations, the term "substantially pure" means that the preparation is essentially free of other substances to an extent practical and appropriate for its intended use. In particular, a protein preparation is substantially pure if it is sufficiently free from other biological constituents so as to be useful in, for example, generating antibodies, sequencing, or producing pharmaceutical preparations. By techniques well known in the art, substantially pure proteins or peptides may be produced in light of the nucleic acid and amino acid sequences disclosed herein. In particular, in light of the nucleic acid and amino acid sequences disclosed herein, one of ordinary skill in the art may, by application or serial application of well-known methods including HPLC or immuno-affinity chromatography or electrophoretic separation, obtain proteins or peptides of any generally feasible purity. Preferably, but not necessarily, "substantially pure"

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preparations include at least 60% by weight (dry weight) the compound of interest. More preferably the preparation is at least 75% or 90%, and most preferably at least 99%, by weight the compound of interest. Purity can be measured by any appropriate method, e.g., column chromatography, gel electrophoresis, or HPLC analysis. With
5 respect to proteins, including antibodies, if a preparation includes two or more different compounds of interest (e.g., two or more different antibodies, immunogens, functional domains, or other polypeptides of the invention), a "substantially pure" preparation is preferably one in which the total weight (dry weight) of all the compounds of interest is at least 60% of the total dry weight. Similarly, for such
10 preparations containing two or more compounds of interest, it is preferred that the total weight of the compounds of interest be at least 75%, more preferably at least 90%, and most preferably at least 99%, of the total dry weight of the preparation. Finally, in the event that the protein of interest is mixed with one or more other proteins (e.g., serum albumin) or compounds (e.g., diluents, excipients, salts,
15 polysaccharides, sugars, lipids) for purposes of administration, stability, storage, and the like, such other proteins or compounds may be ignored in calculation of the purity of the preparation.

Isolated nucleic acid. As used herein, an "isolated nucleic acid" is a ribonucleic acid, deoxyribonucleic acid, or nucleic acid analog comprising a
20 polynucleotide sequence that is isolated or separate from sequences that are immediately contiguous (one on the 5' end and one on the 3' end) in the naturally occurring genome of the organism from which it is derived. The term therefore includes, for example, a recombinant nucleic acid which is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a
25 prokaryote or eukaryote; or which exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequences and/or including exogenous regulatory elements.

Substantially identical sequence. As used herein, a "substantially identical" amino acid sequence is an amino acid sequence which differs only by conservative amino acid substitutions, for example, substitution of one amino acid for another of the same class (e.g., valine for glycine, arginine for lysine, etc.) or by one or more non-conservative substitutions, deletions, or insertions located at positions of the amino acid sequence which do not destroy the function of the protein (assayed, e.g., as described herein). Preferably, such a sequence is at least 85%, more preferably 90%, and most preferably 95% identical at the amino acid level to the sequence of the protein or peptide to which it is being compared. For nucleic acids, the length of comparison sequences will generally be at least 50 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably 110 nucleotides. A "substantially identical" nucleic acid sequence codes for a substantially identical amino acid sequence as defined above.

Transformed cell. As used herein, a "transformed cell" is a cell into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a nucleic acid molecule of interest. The nucleic acid of interest will typically encode a peptide or protein. The transformed cell may express the sequence of interest or may be used only to propagate the sequence. The term "transformed" may be used herein to embrace any method of introducing exogenous nucleic acids including, but not limited to, transformation, transfection, electroporation, microinjection, viral-mediated transfection, and the like.

Operably joined. As used herein, a coding sequence and a regulatory region are said to be "operably joined" when they are covalently linked in such a way as to place the expression or transcription of the coding sequence under the influence or control of the regulatory region. If it is desired that the coding sequences be translated into a functional protein, two DNA sequences are said to be operably joined if induction of promoter function results in the transcription of the coding sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the regulatory region to direct the transcription of the coding sequences, or (3) interfere with the ability of the

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corresponding RNA transcript to be translated into a protein. Thus, a regulatory region would be operably joined to a coding sequence if the regulatory region were capable of effecting transcription of that DNA sequence such that the resulting transcript might be translated into the desired protein or polypeptide.

5 Stringent hybridization conditions. Stringent hybridization conditions is a term of art understood by those of ordinary skill in the art. For any given nucleic acid sequence, stringent hybridization conditions are those conditions of temperature, chaotropic acids, buffer, and ionic strength which will permit hybridization of that nucleic acid sequence to its complementary sequence and not to substantially different
10 sequences. The exact conditions which constitute "stringent" conditions, depend upon the nature of the nucleic acid sequence, the length of the sequence, and the frequency of occurrence of subsets of that sequence within other non-identical sequences. By varying hybridization conditions from a level of stringency at which non-specific hybridization occurs to a level at which only specific hybridization is observed, one of
15 ordinary skill in the art can, without undue experimentation, determine conditions which will allow a given sequence to hybridize only with complementary sequences. Suitable ranges of such stringency conditions are described in Krause and Aaronson (1991). Hybridization conditions, depending upon the length and commonality of a sequence, may include temperatures of 20°C-65°C and ionic strengths from 5x to 0.1x
20 SSC. Highly stringent hybridization conditions may include temperatures as low as 40-42°C (when denaturants such as formamide are included) or up to 60-65°C in ionic strengths as low as 0.1x SSC. These ranges, however, are only illustrative and, depending upon the nature of the target sequence, and possible future technological developments, may be more stringent than necessary. Less than stringent conditions
25 are employed to isolate nucleic acid sequences which are substantially similar, allelic or homologous to any given sequence.

Selectively binds. As used herein with respect to antibodies, an antibody is said to "selectively bind" to a target if the antibody recognizes and binds the target of interest but does not substantially recognize and bind other molecules in a sample,
30 e.g., a biological sample, which includes the target of interest. That is, the antibody

must bind to its target with sufficient specificity so as to distinguish the target from essentially all of molecules which would reasonably be present in a biological sample including the target.

II. The Presenilins and Presenilin-Interacting Proteins

5 The present invention is based, in part, upon the discovery of a family of mammalian genes which, when mutated, are associated with the development of Alzheimer's Disease. The discovery of these genes, designated presenilin-1 (PS1) and presenilin-2 (PS2), as well as the characterization of these genes, their protein
10 products, mutants, invertebrate homologues, and possible functional roles, are described in PCT Publication WO96/34099. The present invention is further based, in part, upon the discovery of a group of proteins which interact with the presenilins under physiological conditions and which, therefore, are believed to be involved in the biochemical pathways which are altered in Alzheimer's Disease. These proteins are referred to herein as presenilin-interacting (PS-interacting) proteins. Because
15 mutations in the presenilins are known to be causative of Alzheimer's Disease, each of the PS-interacting genes and proteins disclosed and described herein presents a novel target for therapeutic intervention in Alzheimer's Disease. That is, modulation of the interactions of these proteins with the presenilins, or modulation of the interactions of at least the PS-interacting domains of these PS-interacting proteins with at least the
20 interacting domains of the presenilins, provides a means of modulating the activity and/or availability of the presenilins, or of modulating the activity and/or availability of the PS-interacting proteins. Furthermore, as aberrations in the interactions of mutant presenilins with one or more of these PS-interacting proteins is causative of Alzheimer's Disease, mutations in one or more of these PS-interacting proteins are
25 also likely to be causative of Alzheimer's Disease. Therefore, each of the PS-interacting genes and proteins disclosed and described herein presents a novel target for diagnosis of forms of familial and/or sporadic Alzheimer's Disease with an etiology independent of mutations in the presenilins. Finally, as described more fully below, the PS-interacting genes and proteins described and disclosed herein provide
30 for new assays for compounds which affect the interactions of the presenilins and PS-

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interacting proteins, assays for other members of the biochemical pathways involved in the etiology of Alzheimer's Disease, and new cell lines and transgenic animal models for use in such assays.

5 1. Presenilin Processing

Employing the antibodies and protein-binding assays described and/or enabled in PCT Publication WO96/34099, the processing and protein-protein interactions of both normal and mutant presenilins were investigated. It was found that mutations in the presenilins appear to lead to changes in both their intracellular
10 processing (e.g., endoproteolytic cleavage, ubiquitination, and clearance) and their intracellular interactions with other proteins expressed in human brain. As described below, knowledge of presenilin processing and interactions, and particularly changes in mutant presenilin processing and interactions, provides for new diagnostic and therapeutic targets for Alzheimer's Disease and related disorders.

15 Western blot analysis suggests that the normal presenilins undergo proteolytic cleavage to yield characteristic N- and C-terminal fragments. As noted above, the normal presenilin proteins have an expected molecular mass of 47-51 kDa depending, in part, upon mRNA splice variations, electrophoretic conditions, etc. Analysis of Western blots suggests, however, that the normal presenilin proteins
20 undergo proteolytic cleavage to yield an approximately 35 kDa N-terminal fragment and an approximately 18 kDa C-terminal fragment. In particular, Western blots bearing lysates from wild-type native human fibroblasts, human neocortical brain tissue from control subjects, and neocortical brain tissue from non-transgenic and PS1 transgenic mice using antibodies ("14.2") recognizing PS1-specific residues 1-25 at
25 the N-terminus reveal the presence of a strong immunoreactive band of approximately 35 kDa and, after longer exposures, a weaker band of approximately 45 kDa which presumably represents the full-length PS1 protein. Antibodies ("520") directed at residues 304-318 at the apex of the TM6→7 loop of PS1, and antibodies ("4627") directed at residues 457-467 in the C-terminus of PS1, both recognize the same strong
30 band of approximately 18 kDa. Antibodies 520 also recognize a weak band of 45 kDa

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coincident with the PS1 band detected by 14.2. Sequencing of the major C-terminal fragment from PS1-transfected human embryonic kidney cells (HEK 293) showed that the principal endoproteolytic cleavage occurs near M298 in the proximal portion of the TM6→7 loop, possibly by enzymes other than the proteasome. These
5 observations suggest that an endoproteolytic cleavage event occurs near the junction of exons 9 and 10 of PS1. Full length PS1 in these cells is quickly turned over ($t_{1/2} < 60$ min.) by the proteasome.

To determine whether mutations in the presenilin proteins result in alterations of their proteolytic cleavage, Western blots containing lysates of fibroblast
10 and neocortical brain homogenates from normal subjects and subjects carrying PS1 mutations were investigated with the PS1 specific antibody Ab 14.2. In fibroblasts, there were no obvious differences in the relative intensities of the protein bands when lysates from heterozygous carriers of the PS1 mutations were compared with normal homozygotes. In contrast, there appeared to be a difference between PS1 mutation
15 carriers and normals in homogenates of temporal neocortex from AD affected heterozygous carriers of either the PS1 A246E or C410Y mutations (which are located in TM6 and TM7 respectively). In heterozygotes, a strongly immunoreactive band of approximately 45 kDa was detected which initially appeared to correspond to the full-length PS1 protein. Further analysis, however, revealed that this band represents an
20 alternatively processed presenilin product. A similar band corresponding to this mutant processed PS1 was observed in neocortical homogenates from some sporadic late-onset AD patients. These data suggest that (1) some pathogenic PS1 mutations associated with early-onset AD alter the way in which the presenilins are processed through endoproteolytic and proteasome pathways and (2) the presenilin proteins, and
25 changes in the processing of the presenilins in the brain, are also implicated in late-onset and sporadic AD.

2. Presenilin-Interacting Proteins

In order to identify proteins which may bind to or otherwise interact with
30 the presenilins in vivo, a yeast two-hybrid system was used as described below

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(Example 1). In particular, because mutations in the TM6→7 loop domains are known to be causative of AD, a yeast two-hybrid system was used to identify cellular proteins which may interact with normal and mutant presenilin TM6→7 loop domains. Yeast two-hybrid studies were also done with cDNAs corresponding to the C-terminal 18 kDa endoproteolytic cleavage fragment, and with cDNAs corresponding to the TM1→2 intraluminal loop domain, which is also the site of the FAD associated Y115H missense mutation. In brief, cDNA sequences encoding the TM6→7 loop (i.e., residues 266 to 409 of PS1) were ligated in-frame to the GAL4 DNA-binding domain in the pAS2-1 yeast expression plasmid vector (Clontech). This plasmid was then co-transformed into *S. cerevisiae* strain Y190 together with a library of human brain cDNAs ligated into the pACT2 yeast expression vector bearing the GAL4 activation domain (Clontech). After appropriate selection and re-screening, a number of clones were recovered and sequenced bearing human brain cDNAs encoding peptides which interacted with the normal presenilin TM6→7 domain. To determine whether these presenilin interactions would be modified by AD related mutations within the TM6→7 loop, the yeast two-hybrid system was again used with TM6→7 loop peptides containing the L286V, the L392V, and the exon 10 splicing mutants. When these mutant constructs were used as "bait" to re-screen the brain cDNA:GAL4 activation domain library, some but not all of the brain cDNA sequences which interacted with the normal presenilin were recovered. In addition, several new clones were identified which interacted with the mutant but not the normal presenilins. The clones corresponding to the PS-interacting proteins with the highest presenilin affinity are described in Example 1 and below.

PS-interacting proteins, particularly those which interact selectively with either the normal or mutant presenilins, provide new targets for the identification of useful pharmaceuticals, new targets for diagnostic tools in the identification of individuals at risk, new sequences for the production of transformed cell lines and transgenic animal models, and new bases for therapeutic intervention in Alzheimer's Disease. In particular, the onset of AD may be associated with aberrant interactions between mutant presenilin proteins and normal forms of PS-interacting proteins such

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as those identified using the methods described herein. These changes may increase or decrease interactions present with normal PS1 or may cause interaction with a novel mutation-specific PS-interacting protein. In addition, however, aberrant interactions may result from normal presenilins binding to mutant forms of the PS-interacting proteins and, therefore, mutations in the PS-interacting proteins may also be causative of AD.

A. The S5a Subunit of the 26S Proteasome

Two overlapping clones have been identified as representing a portion of the human protein alternatively known as Antisecretory Factor ("ASF") or the Multiubiquitin chain-binding S5a subunit of the 26S proteasome ("S5a"). These clones, which together include residues 70-377 of S5a, were shown to interact with the normal presenilin TM6→7 loop domain but only weakly with two TM6→7 loop domain mutants tested (L286V, L392V). The PS1:S5a interaction was confirmed by co-immunoprecipitation studies, and immunocytochemical studies showed S5a and PS1 are expressed in contiguous intracellular compartments in brain cells typically affected by AD.

The interaction between PS1 and the proteasome could be relevant to the pathogenesis of Alzheimer's Disease (AD) through several possible mechanisms. First, most mammalian cells seem to maintain very low levels of the PS1 holoprotein. A notable exception to this are cells expressing the PS1 $\Delta 290-319$ splicing mutation, which results in a mutant PS1 holoprotein which is not endoproteolytically cleaved and which is, therefore, readily detectable. In the case of the $\Delta 290-319$ splicing mutation at least, the presence of the mutant PS1 holoprotein, or the absence or reduction in the 35 kDa N-terminal and 18 kDa C-terminal fragments, appears sufficient to cause AD. It is possible, therefore, that even very subtle changes in the turnover of the mutant PS1 holoprotein might have significant pathophysiological effects. Thus, mutations in either the presenilins or S5a which perturb the PS1:S5a interaction in the mammalian CNS may cause the presenilin holoprotein to be aberrantly processed and cause AD. Therefore, modulation of presenilin proteolytic pathways might be applied therapeutically to enhance removal of mutant holoprotein.

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To assess a potential in vivo relationship between PS1 and the S5a subunit of the 26S proteasome, the effects of proteasome inhibitors on PS1 metabolism were investigated. Short term organotypic cultures of neonatal rat hippocampus and carcinoma of colon (CaCo2) cells (which express high levels of both PS1 and PS2) were administered either the specific, reversible proteasome inhibitor N-acetyl-leuciny-leuciny-norleuciny-H (LLnL) (Rock et al., 1994), or the specific irreversible proteasome inhibitor lactacystin (Fenteany et al., 1995). Both agents caused an increase in the steady state levels of PS1 holoprotein. Both agents also prolonged the half-life of the PS1 holoprotein in pulse chase experiments in hippocampal slices from ~15 minutes to ~35 minutes. As noted above, the PS1 holoprotein appears to be rapidly turned over in normal cells. However, even after four hours of metabolic labelling, neither of the proteasome inhibitors affected the level of the 35 kDa N-terminal PS1 fragment, or resulted in the appearance of novel species. These studies imply that the majority of the PS1 holoprotein is catabolized directly via a rapid, proteasome dependent pathway in a manner similar to several other integral membrane proteins (e.g. Sec61 and CFTR). On the other hand, because the ~35 kDa and ~18 kDa terminal fragments are still produced in the presence of proteasome inhibitors, this endoproteolytic cleavage of PS1 is probably not mediated by the proteasome pathway. Therefore, it appears that at least two proteolytic pathways act upon the PS1 holoprotein.

An alternate possibility is that mutant PS1:S5a interactions may modify the function or the cellular regulation of S5a. To address this possibility, S5a levels were examined by Western blotting of lysates from postmortem temporal neocortex from non-AD neurologic controls (n = 8), sporadic AD (n = 8) and PS1-linked FAD (n = 4). In the majority of non-AD brains, polyclonal anti-S5a antibodies specifically detected an S5a species with Mr of ~ 50 kDa, which could be abolished by preabsorption of the antibody with recombinant His₆-S5a or with extracts of myc-S5a transfected cells. In a subset of these control cases an additional S5a reactive band was observed at ~34 kDa. In contrast, in tissue from all subjects with sporadic late onset AD, the predominant S5a reactive species was observed at ~ 40 kDa which was

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not seen in control tissue. The origin, and the functional significance of this altered electrophoretic mobility is unclear but indicates that S5a processing is altered in AD brains, irrespective of whether the AD is presenilin-linked or sporadic.

Thus, the presenilin-proteasome interaction appears significant in several
5 respects. First, the facts that the normal presenilin TM6→7 loop domain interacts with the S5a protein, that the mutant presenilin TM6→7 loop domains fail to interact (or interact very weakly) with the S5a protein, that presenilins bearing mutations in the TM6→7 loop domain appear to be differently cleaved and multiubiquitinated, that
10 proteasomes are known to be involved in the cleavage and clearance of a variety of proteins (particularly multiubiquitinated proteins), that inhibition of proteasome activity inhibits cleavage of the presenilin holoproteins, and that S5a processing is altered in AD brains, all suggest (1) that the S5a subunit and the 26S proteasome are involved in the normal processing of the presenilins and that mutations which disrupt this normal interaction may be responsible for the abnormal processing observed in
15 TM6→7 loop domain mutants; or (2) that the presenilin-proteasome interaction may modulate the activity of PS1, S5a, or both, with or without involving proteasome-mediated presenilin processing; or (3) that modulation of the normal quality control function of proteasome-mediated degradation of misfolded or mutant membrane proteins trafficking through the ER and Golgi (such as APP, Notch, or Prion proteins),
20 and of misfolded, mutant, or ubiquitinated cytoplasmic proteins (including structural proteins such as tau, and short lived, proteasome processed signaling molecules such as NFkB). Thus, defective proteasome function might selectively cause these proteins (especially βAPP, tau, Prion) to be aberrantly metabolized. The latter would lead to the accumulation of neurotoxic, amyloidogenic protease-resistant derivatives such as
25 Aβ and PrPsc, the accumulation of neurofibrillary tangles, and defective intracellular signaling functions. In support of these hypotheses, it should be noted that failure to clear hyperubiquitinated phosphorylated tau and other microtubule associated proteins is a prominent feature of Alzheimer's Disease (Kosik and Greenberg, 1994), suggesting a possible link between TM6→7 loop domain mutants, presenilin-
30 proteasome interactions, tau-proteasome interactions, and the neurofibrillary tangles

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of tau protein in AD brains. Finally, proteasomes are known to be capable of degrading APP and of binding the A β peptides which are associated with Alzheimer's Disease, suggesting a possible link between TM6 \rightarrow 7 loop domain mutants, presenilin-proteasome interactions, APP-proteasome interactions, and the amyloid
5 plaques characteristic of AD brains. Furthermore, administration of proteasome inhibitors such as LLnL and Lactacystin cause severe disturbances in β APP metabolism with increases in intracellular immature N-glycosylated β APP, and the secretion of much larger amounts of A β_{42} isoforms into the media (Klaffki, et al., 1996).

10 Therefore, presenilin processing and the presenilin-proteasome interaction are clear targets for the diagnosis as well as therapeutic intervention in AD. Thus, as described below, assays may now be provided for drugs which affect the proteasome-mediated cleavage of the presenilins, which affect the alternative endoproteolytic cleavage and ubiquitination of the mutant presenilins, or which otherwise affect the
15 processing and trafficking of the presenilins or the S5a subunit of the proteasome. In addition, as mutations in the 26S proteasome which disrupt the normal processing of the presenilins are likely to be causative of Alzheimer's Disease, additional diagnostic assays are provided for detecting mutations in the S5a or other subunits of the proteasome. Finally, additional transformed cell lines and transgenic models may
20 now be provided which have been altered by the introduction of a normal or mutant sequence encoding at least a functional domain of the proteasome. The appearance of abnormal electrophoretic forms of S5a (and/or other proteasome subunits) in biologic tissues and fluids can be used as a clinical test for diagnosis and monitoring of disease activity in subjects with sporadic forms of AD.

25 B. GT24: A Protein with "Armadillo" Repeats

Another PS-interacting protein, designated GT24, was identified from several over-lapping clones obtained using a PS1₂₆₆₋₄₀₉ domain as bait in the yeast two-hybrid system and a human adult brain cDNA library. Six longer GT24 clones of
~3.8 kb in size were subsequently obtained by screening of conventional cDNA
30 libraries. The open reading frame within the longest GT24 clone obtained to date

(Accession number U81004) suggests that GT24 is a protein of at least 1040 amino acids with a unique N-terminus, and considerable homology to several armadillo (arm) repeat proteins at its C-terminus. Thus, for example, residues 440-862 of GT24 (numbering from Accession number U81004) have 32-56% identity ($p=1.2e^{-133}$) to residues 440-854 of murine p120 protein (Accession number Z17804), and residues 367-815 of GT24 have 26-42% identity ($p=0.0017$) to residues 245-465 of the D. melanogaster armadillo segment polarity protein (Accession number P18824). The GT24 gene maps to chromosome 5p15 near the anonymous microsatellite marker D5S748 and the Cri-du-Chat syndrome locus.

Hybridization of unique 5' sequences of GT24 to Northern blots reveals that the GT24 gene is expressed as a range of transcripts varying in size between ~3.9 and 5.0 kb in several regions of human brain, and in several non-neurologic tissues such as heart. In addition, in situ hybridization studies using a 289 bp single copy fragment from the 5' end of GT24 in four month old murine brain reveal GT24 transcription closely parallels that of PS1, with robust expression in dentate and hippocampal neurons, in scattered neocortical neurons, and in cerebellar Purkinje cells. In day E13 murine embryos, GT24 is widely expressed at low levels, but is expressed at somewhat higher levels in somites and in the neural tube. A physiological in vivo interaction between GT24 and PS1 is supported by co-immunoprecipitation studies in HEK293 cells transiently transfected with a wild type human PS1 cDNA, a c-myc-tagged cDNA encoding residues 484-1040 of GT24 (including the C-terminal arm repeats), or both cDNAs. Cell lysates were immunoprecipitated with anti-PS1 antibodies and then investigated for the presence of the myc-GT24 protein by immuno-blotting. In PS1/myc-GT24 double transfected cells, the immunoprecipitates contained a robust anti-myc reactive band of Mr ~60 kDa, which co-migrated with a myc-GT24 control. In cells transfected with myc-GT24 only, a very weak band was detected after long exposures, presumably reflecting interaction of the myc-GT24 with low levels of endogenous PS1. No myc-reactive bands were detected in cells transfected with PS1 alone, or in any of the transfected cells immunoprecipitated with pre-immune serum. Taken together, these

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observations strongly suggest that the observed PS1:GT24 interaction is physiologically relevant.

To explore whether mutations in the TM6-TM7 loop of PS1 might influence the PS1:GT24 interaction, we employed quantitative liquid β -galactosidase assays to directly compare the yeast-two-hybrid interaction of the C-terminal residues 499-1040 of GT24 with wildtype and mutant PS1₂₆₆₋₄₀₉. These studies revealed that the interaction of GT24₄₉₉₋₁₀₄₀ with a L286V mutant PS1 domain was not significantly different from the interaction with the corresponding wild type PS1 domain. In contrast, there was a significant reduction in the GT24₄₉₉₋₁₀₄₀ interaction with the L392V mutant PS1 construct. The absence of an effect of the L286V mutation, and the presence of an effect with the L392V mutation, may suggest that some mutations may effect PS1:GT24 binding, while others may modulate the PS1 response to GT24 binding.

The PS1:GT24 interaction could support several functions. The arm repeat motif of GT24 has been detected in several proteins with diverse functions including β -catenin and its invertebrate homologue armadillo, plakoglobin, p120, the adenomatous polyposis coli (APC) gene, suppressor of RNA polymerase 1 in yeast (SRP1), and smGDS. For example, β -catenin, p120 and plakoglobin play an essential role in intercellular adhesion. β -catenin/armadillo is involved in transduction of wingless/Wnt signals during cell fate specification, and β -catenin and p120 may play a role in other receptor mediated signal transduction events including responses to trophic factors such as PDGF, EGF, CSF-1 and NGF.

If the PS1:GT24 interaction is part of intercellular signaling pathways for trophic factors, or is involved in cell-cell adherence, disruption of the interaction may be involved in the neurodegenerative processes in PS-linked FAD brains, and in the increased sensitivity of PS1 or PS2 transfected cells to apoptosis (Wolozin et al., 1996). It is of note that at least one arm protein, smGDS, stimulates GDP/GTP exchange on intracellular G-proteins (Kikuchi et al. 1992; Borguski et al., 1993), and that mutant forms of both β APP and PS2 are thought to activate programmed cell

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death pathways through mechanisms involving heterotrimeric GTP/GDP proteins (Wolozin et al, 1996; Okamoto, et al., 1995; Yamatsuji, et al, 1996).

The interaction between PS1 and GT24 may also be involved in some of the developmental phenotypes associated with homozygous PS1 knockouts in mice
5 such as failed somitogenesis of the caudal embryo, short tail, and fatal cerebral hemorrhage at around day E13.5 (Wong et al., 1996). The resemblance of these skeletal phenotypes to those associated with null mutations in PAX1 and Notch, and the apparent suppressor effect of mutations in sel12 on Notch/lin12 mediated signaling in C. elegans suggest that the PS proteins function in the Notch signaling
10 pathway. In addition, mice homozygous for a knockout of the Wnt-3a gene (Takada et al., 1994), and murine homozygotes for a spontaneous mutation, "vestigial tail" or vt, in the Wnt-3a gene (Greco et al., 1996), have skeletal phenotypes of defective caudal somite and tail bud formation. The Wnt-3a knockouts are embryonic lethal by day 12.5. These phenotypes are similar to those of homozygous knockouts of the
15 murine PS1 gene (Wong et al., 1996). The observation that GT24 binds to PS1, is expressed in embryonic somites, and contains the armadillo repeat motif of other proteins used in the downstream signaling in the Wingless/Wnt pathway suggests that PS1 is a downstream element in the GT24-Wingless/Wnt pathway. This can be exploited to create a bioassay for drugs affecting the GT24-PS1 interaction directly, or
20 affecting upstream or downstream components of that interaction pathway, and can therefore be used to monitor the effects of presenilin mutations. For example, cells transfected with normal or mutant presenilins may be exposed to soluble Wnt-3a protein (or other Wnt proteins such as Wnt-1) and assayed for changes which are specific to the Wingless/Wnt signaling pathway, or for any of the other changes
25 described herein for cell assays (e.g., intracellular ion levels, A β processing, apoptosis, etc.).

Thus, the GT24 protein also presents new targets for diagnosis as well as therapeutic intervention in AD. For example, as mutations in the GT24 protein may also be causative of Alzheimer's Disease, additional diagnostic assays are provided for
30 detecting mutations in these sequences. Similarly, additional transformed cell lines

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and transgenic models may now be provided which have been altered by introduction of a normal or mutant nucleic acid encoding at least a functional domain of the GT24 protein, and particularly the functional domains (e.g., residues 70-377) which interact with the presenilins. Such transformed cells and transgenics will have utility in assays for compounds which modulate the presenilin-GT24 interactions.

C. p0071: A Protein with "Armadillo" Repeats

Another independent clone isolated in the initial screening with the wild type PS1₂₆₆₋₄₀₉ "bait" also encodes a peptide with C-terminal arm repeats (clone Y2H25, Accession number U81005). A longer cDNA sequence corresponding to the Y2H25 clone has been deposited with GenBank as human protein p0071 (Accession number X81889) and is reproduced herein as SEQ ID NO: 5. Clone Y2H25 corresponds essentially to nucleotide positions 1682-1994 of SEQ ID NO: 5. Comparison of the predicted sequence of the Y2H25/p0071 ORF with that of GT24 confirms that they are related proteins with 47% overall amino acid sequence identity, and with 70% identity between residues 346-862 of GT24 and residues 509-1022 of p0071. This suggests that PS1 interacts with a novel class of arm repeat containing proteins. The broad ~4.5 kb hybridization signal obtained on Northern blots with the unique 5' end of GT24 could reflect either alternative splicing/polyadenylation of GT24 or, less likely, the existence of additional members of this family with higher degrees of N-terminal homology to GT24 than p0071. Cells transformed with these sequences, or transgenic animals including these sequences, will have additional utility as animal models of AD and for use in screening for compounds which modulate the action of normal and mutant presenilins.

D. Rab 11

One clone (Y2H9), disclosed herein as SEQ ID NO: 5, was identified as interacting with the normal PS1 TM6→7 loop domain and appears to correspond to a known gene, Rab11, available through Accession numbers X56740 and X53143. Rab11 is believed to be involved in protein/vesicle trafficking in the ER/Golgi. Note the possible relationship to processing of membrane proteins such as β APP and Notch

with resultant overproduction of toxic A β peptides (especially neurotoxic A $\beta_{1-42/43}$ isoforms) (Scheuner, et al, 1995).

E. Retinoid X Receptor- β

One clone (Y2H23b), disclosed herein as SEQ ID NO: 6, was identified as
5 interacting with the normal PS1 TM6 \rightarrow 7 loop domain and appears to correspond to a known gene, known variously as the retinoid X receptor- β , nuclear receptor co-regulator, or MHC Class I regulatory element, and is available through Accession numbers M84820, X63522 and M81766. This gene is believed to be involved in intercellular signaling, suggesting a possible relationship to the intercellular signaling
10 function mediated by *C. elegans* sel12 and Notch/lin-12 (transcription activator).

F. Cytoplasmic Chaperonin

One clone (Y2H27), disclosed herein as SEQ ID NO: 8, was identified as interacting with the normal PS1 TM6 \rightarrow 7 loop domain and appears to correspond to a known gene, a cytoplasmic chaperonin containing TCP-1, available through
15 Accession numbers U17104 and X74801.

G. Clone Y2H35

One clone (Y2H35), disclosed herein as SEQ ID NO: 7, was identified as interacting with the normal PS1 TM6 \rightarrow 7 loop domain and appears to correspond to a sequence that codes for a protein of unknown function, available through Accession
20 number R12984, but which displays evolutionary conservation in yeast sequences.

H. Clone Y2H171

One clone (Y2H171), disclosed herein as SEQ ID NO: 9, was identified as interacting with the normal PS1 TM6 \rightarrow 7 loop domain and appears to correspond to a known expressed repeat sequence available through Accession number D55326.

25 I. Clone Y2H41

One clone (Y2H41) was identified which reacts strongly with the TM6 \rightarrow 7 loop domains of both PS1 and PS2 as well as the mutant loop domains of PS1. The sequence, disclosed as SEQ ID NO: 10, shows strong homology to an EST of unknown function (Accession number T64843).

III. Preferred Embodiments

Based, in part, upon the discoveries disclosed and described herein, the following preferred embodiments of the present invention are provided.

5

1. Isolated Nucleic Acids

In one series of embodiments, the present invention provides isolated nucleic acids corresponding to, or relating to, the nucleic acid sequences disclosed herein, which encode at least the PS-interacting domain of a PS-interacting protein.

10 As described more fully below, the disclosed and enabled sequences include normal sequences from humans and other mammalian species, mutant sequences from humans and other mammalian species, homologous sequences from non-mammalian species such as Drosophila and C. elegans, subsets of these sequences useful as probes and PCR primers, subsets of these sequences encoding fragments of the PS-interacting
15 proteins or corresponding to particular structural domains or polymorphic regions, complementary or antisense sequences corresponding to fragments of the PS-interacting protein genes, sequences in which the PS-interacting protein coding regions have been operably joined to exogenous regulatory regions, and sequences encoding fusion proteins in which portions of the PS-interacting proteins are fused to
20 other proteins useful as markers of expression, as "tags" for purification, or in screens and assays for other proteins which interact with the PS-interacting proteins.

Thus, in a first series of embodiments, isolated nucleic acid sequences are provided which encode at least a PS-interacting domain of a normal or mutant version of a PS-interacting protein. Examples of such nucleic acid sequences are disclosed
25 herein as SEQ ID NOs: 1, 3, and 5-10. In addition, given the sequences of the PS-interacting domains of the PS-interacting proteins disclosed herein, one of ordinary skill in the art is clearly enabled to obtain the entire genomic or cDNA sequence encoding the entire PS-interacting proteins. Thus, for example, based upon the initial clone of the GT24 protein obtained using the yeast two-hybrid system (Example 1),
30 the larger GT24 clone disclosed as SEQ ID NO: 3 was obtained by standard methods

known in the art. Complete cDNA or genomic clones of each of the genes encoding the disclosed sequences may be similarly obtained by one of ordinary skill in the art. Therefore, the present invention provides complete genomic sequences as well as cDNA sequences corresponding to the PS-interacting protein genes of the invention.

5 Alternatively, the nucleic acids of the invention may comprise recombinant genes or "minigenes" in which all or some introns of the PS-interacting protein genes have been removed, or in which various combinations of introns and exons and local cis-acting regulatory elements have been engineered in propagation or expression constructs or vectors. For purposes of reducing the size of a recombinant PS-

10 interacting protein gene, a cDNA gene may be employed, or various combinations of introns and untranslated exons may be removed from a DNA construct. These and many variations on these embodiments are now enabled by the identification and description of the PS-interacting proteins provided herein.

In addition to the disclosed PS-interacting protein and gene sequences, one

15 of ordinary skill in the art is now enabled to identify and isolate nucleic acids representing PS-interacting genes or cDNAs which are allelic to the disclosed sequences or which are heterospecific homologues. Thus, the present invention provides isolated nucleic acids corresponding to these alleles and homologues, as well as the various above-described recombinant constructs derived from these sequences.

20 by means which are well known in the art. Briefly, one of ordinary skill in the art may now screen preparations of genomic or cDNA, including samples prepared from individual organisms (e.g., human AD patients or their family members) as well as bacterial, viral, yeast or other libraries of genomic or cDNA, using probes or PCR primers to identify allelic or homologous sequences. Because it is desirable to

25 identify mutations in the PS-interacting proteins which may contribute to the development of AD or other disorders, because it is desirable to identify polymorphisms in the PS-interacting proteins which are not pathogenic, and because it is also desirable to create a variety of animal models which may be used to study AD and screen for potential therapeutics, it is particularly contemplated that additional

30 PS-interacting protein sequences will be isolated from other preparations or libraries

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of human nucleic acids and from preparations or libraries from animals including rats, mice, hamsters, guinea pigs, rabbits, dogs, cats, goats, sheep, pigs, and non-human primates. Furthermore, PS-interacting protein homologues from yeast or invertebrate species, including C. elegans and other nematodes, as well as Drosophila and other
5 insects, may have particular utility for drug screening.

Standard hybridization screening or PCR techniques may be employed (as used, for example, in the identification of the mPS1 gene disclosed in PCT Publication WO96/34099) to identify and/or isolate such allelic and homologous sequences using relatively short PS-interacting protein gene sequences. The
10 sequences may include 8 or fewer nucleotides depending upon the nature of the target sequences, the method employed, and the specificity required. Future technological developments may allow the advantageous use of even shorter sequences. With current technology, sequences of 9-50 nucleotides, and preferably about 18-24 are preferred. These sequences may be chosen from those disclosed herein, or may be
15 derived from other allelic or heterospecific homologues enabled herein. When probing mRNA or screening cDNA libraries, probes and primers from coding sequences (rather than introns) are preferably employed, and sequences which are omitted in alternative splice variants typically are avoided unless it is specifically desired to identify those variants. Allelic variants of the PS-interacting protein genes
20 may be expected to hybridize to the disclosed sequences under stringent hybridization conditions, as defined herein, whereas lower stringency may be employed to identify heterospecific homologues.

In another series of embodiments, the present invention provides for isolated nucleic acids which include subsets of the PS-interacting protein sequences or
25 their complements. As noted above, such sequences will have utility as probes and PCR primers in the identification and isolation of allelic and homologous variants of the PS-interacting protein genes. Subsequences corresponding to polymorphic regions of the PS-interacting proteins, will also have particular utility in screening and/or genotyping individuals for diagnostic purposes, as described below. In
30 addition, and also as described below, such subsets will have utility for encoding (1)

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fragments of the PS-interacting proteins for inclusion in fusion proteins, (2) fragments which comprise functional domains of the PS-interacting proteins for use in binding studies, (3) fragments of the PS-interacting proteins which may be used as immunogens to raise antibodies against the PS-interacting proteins, and (4) fragments of the PS-interacting proteins which may act as competitive inhibitors or as mimetics of the PS-interacting proteins to inhibit or mimic their physiological functions. Finally, such subsets may encode or represent complementary or antisense sequences which can hybridize to the PS-interacting protein genes or PS-interacting protein mRNA transcripts under physiological conditions to inhibit the transcription or translation of those sequences. Therefore, depending upon the intended use, the present invention provides nucleic acid subsequences of the PS-interacting protein genes which may have lengths varying from 8-10 nucleotides (e.g., for use as PCR primers) to nearly the full size of the PS-interacting protein genomic or cDNAs. Thus, the present invention provides isolated nucleic acids comprising sequences corresponding to at least 8-10, preferably 15, and more preferably at least 20 consecutive nucleotides of the PS-interacting protein genes, as disclosed or otherwise enabled herein, or to their complements. As noted above, however, shorter sequences may be useful with different technologies.

In another series of embodiments, the present invention provides nucleic acids in which the coding sequences for the PS-interacting proteins, with or without introns or recombinantly engineered as described above, are operably joined to endogenous or exogenous 5' and/or 3' regulatory regions. Using the present disclosure and standard genetic techniques (e.g., PCR extensions, targeting gene walking), one of ordinary skill in the art is now enabled to clone the 5' and/or 3' endogenous regulatory regions of any of the disclosed PS-interacting protein genes. Similarly, allelic variants of these endogenous regulatory regions, as well as endogenous regulatory regions from other mammalian homologues, are similarly enabled without undue experimentation. Alternatively, exogenous regulatory regions (i.e., regulatory regions from a different conspecific gene or a heterospecific regulatory region) may be operably joined to the PS-interacting protein coding sequences in order to drive

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expression. Appropriate 5' regulatory regions will include promoter elements and may also include additional elements such as operator or enhancer sequences, ribosome binding sequences, RNA capping sequences, and the like. The regulatory region may be selected from sequences that control the expression of genes of

5 prokaryotic or eukaryotic cells, their viruses, and combinations thereof. Such regulatory regions include, but are not limited to, the lac system, the trp system, the tac system, and the trc system; major operator and promoter regions of phage λ ; the control region of the fd coat protein; early and late promoters of SV40; promoters derived from polyoma, adenovirus, retrovirus, baculovirus, and simian virus; 3-

10 phosphoglycerate kinase promoter; yeast acid phosphatase promoters; yeast alpha-mating factors; promoter elements of other eukaryotic genes expressed in neurons or other cell types; and combinations thereof. In particular, regulatory elements may be chosen which are inducible or repressible (e.g., the β -galactosidase promoter) to allow for controlled and/or manipulable expression of the PS-interacting protein genes in

15 cells transformed with these nucleic acids. Alternatively, the PS-interacting protein coding regions may be operably joined with regulatory elements which provide for tissue specific expression in multicellular organisms. Such constructs are particularly useful for the production of transgenic organisms to cause expression of the PS-interacting protein genes only in appropriate tissues. The choice of appropriate

20 regulatory regions is within the ability and discretion of one of ordinary skill in the art and the recombinant use of many such regulatory regions is now established in the art.

In another series of embodiments, the present invention provides for isolated nucleic acids encoding all or a portion of the PS-interacting proteins in the form of a fusion protein. In these embodiments, a nucleic acid regulatory region

25 (endogenous or exogenous) is operably joined to a first coding region which is covalently joined in-frame to a second coding region. The second coding region optionally may be covalently joined to one or more additional coding regions and the last coding region is joined to a termination codon and, optionally, appropriate 3' regulatory regions (e.g., polyadenylation signals). The PS-interacting protein

30 sequences of the fusion protein may represent the first, second, or any additional

coding regions. The PS-interacting protein sequences may be conserved or non-conserved domains and can be placed in any coding region of the fusion. The non-PS-interacting protein sequences of the fusion may be chosen according to the needs and discretion of the practitioner and are not limited by the present invention. Useful
5 non-PS-interacting protein sequences include, for example, short sequence "tags" such as antigenic determinants or poly-His tags which may be used to aid in the identification or purification of the resultant fusion protein. Alternatively, the non-PS-interacting protein coding region may encode a large protein or protein fragment, such as an enzyme or binding protein which also may assist in the identification and
10 purification of the protein, or which may be useful in an assay such as those described below. Particularly contemplated fusion proteins include poly-His and GST (glutathione S-transferase) fusions which are useful in isolating and purifying the presenilins-interacting proteins, and the yeast two hybrid fusions, described below, which are useful in assays to identify other proteins which bind to or interact with the
15 PS-interacting proteins.

In another series of embodiments, the present invention provides isolated nucleic acids in the form of recombinant DNA constructs in which a marker or reporter gene (e.g., β -galactosidase, luciferase) is operably joined to the 5' regulatory region of a PS-interacting protein gene such that expression of the marker gene is
20 under the control of those regulatory sequences. Using the PS-interacting protein regulatory regions enabled herein, including regulatory regions from human and other mammalian species, one of ordinary skill in the art is now enabled to produce such constructs. As discussed more fully below, such isolated nucleic acids may be used to produce cells, cell lines or transgenic animals which are useful in the identification of
25 compounds which can, directly or indirectly, differentially affect the expression of the PS-interacting proteins.

Finally, the isolated nucleic acids of the present invention include any of the above described sequences when included in vectors. Appropriate vectors include cloning vectors and expression vectors of all types, including plasmids, phagemids,
30 cosmids, episomes, and the like, as well as integration vectors. The vectors may also

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include various marker genes (e.g., antibiotic resistance or susceptibility genes) which are useful in identifying cells successfully transformed therewith. In addition, the vectors may include regulatory sequences to which the nucleic acids of the invention are operably joined, and/or may also include coding regions such that the nucleic acids of the invention, when appropriately ligated into the vector, are expressed as fusion proteins. Such vectors may also include vectors for use in yeast "two hybrid," baculovirus, and phage-display systems. The vectors may be chosen to be useful for prokaryotic, eukaryotic or viral expression, as needed or desired for the particular application. For example, vaccinia virus vectors or simian virus vectors with the SV40 promoter (e.g., pSV2), or Herpes simplex virus or adeno-associated virus may be useful for transfection of mammalian cells including neurons in culture or *in vivo*, and the baculovirus vectors may be used in transfecting insect cells (e.g., butterfly cells). A great variety of different vectors are now commercially available and otherwise known in the art, and the choice of an appropriate vector is within the ability and discretion of one of ordinary skill in the art.

2. Substantially Pure Proteins

The present invention provides for substantially pure preparations of the PS-interacting proteins, fragments of the PS-interacting proteins, and fusion proteins including the PS-interacting proteins or fragments thereof. The proteins, fragments and fusions have utility, as described herein, in the generation of antibodies to normal and mutant PS-interacting proteins, in the identification of proteins (aside from the presenilins) which bind to the PS-interacting proteins, and in diagnostic and therapeutic methods. Therefore, depending upon the intended use, the present invention provides substantially pure proteins or peptides comprising amino acid sequences which are subsequences of the complete PS-interacting proteins and which may have lengths varying from 4-10 amino acids (e.g., for use as immunogens), or 10-100 amino acids (e.g., for use in binding assays), to the complete PS-interacting proteins. Thus, the present invention provides substantially pure proteins or peptides comprising sequences corresponding to at least 4-5, preferably 6-10, and more

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preferably at least 50 or 100 consecutive amino acids of the PS-interacting proteins, as disclosed or otherwise enabled herein.

The proteins or peptides of the invention may be isolated and purified by any of a variety of methods selected on the basis of the properties revealed by their protein sequences. For example, the PS-interacting proteins may be isolated from
5 cells in which the PS-interacting protein is normally highly expressed. Alternatively the PS-interacting protein, fusion protein, or fragment thereof, may be purified from cells transformed or transfected with expression vectors (e.g., baculovirus systems such as the pPbac and pMbac vectors (Stratagene, La Jolla, CA); yeast expression
10 systems such as the pYESHIS Xpress vectors (Invitrogen, San Diego, CA); eukaryotic expression systems such as pcDNA3 (Invitrogen, San Diego, CA) which has constant constitutive expression, or LacSwitch (Stratagene, La Jolla, CA) which is inducible; or prokaryotic expression vectors such as pKK233-3 (Clontech, Palo Alto, CA). In the event that the protein or fragment integrates into the endoplasmic reticulum or
15 plasma membrane of the recombinant cells (e.g., eukaryotic cells), the protein may be purified from the membrane fraction. Alternatively, if the protein aggregates in inclusion bodies within the recombinant cells (e.g., prokaryotic cells), the protein may be purified from whole lysed cells or from solubilized inclusion bodies.

Purification can be achieved using standard protein purification procedures
20 including, but not limited to, gel-filtration chromatography, ion-exchange chromatography, high-performance liquid chromatography (RP-HPLC, ion-exchange HPLC, size-exclusion HPLC, high-performance chromatofocusing chromatography, hydrophobic interaction chromatography, immunoprecipitation, or immunoaffinity purification. Gel electrophoresis (e.g., PAGE, SDS-PAGE) can also be used to isolate
25 a protein or peptide based on its molecular weight, charge properties and hydrophobicity.

A PS-interacting protein, or a fragment thereof, may also be conveniently purified by creating a fusion protein including the desired PS-interacting protein sequence fused to another peptide such as an antigenic determinant or poly-His tag
30 (e.g., QIAexpress vectors, QIAGEN Corp., Chatsworth, CA), or a larger protein (e.g.,

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GST using the pGEX-27 vector (Amrad, USA) or green fluorescent protein using the Green Lantern vector (GIBCO/BRL, Gaithersburg, MD). The fusion protein may be expressed and recovered from prokaryotic or eukaryotic cells and purified by any standard method based upon the fusion vector sequence. For example, the fusion protein may be purified by immunoaffinity or immunoprecipitation with an antibody to the non-PS-interacting protein portion of the fusion or, in the case of a poly-His tag, by affinity binding to a nickel column. The desired PS-interacting protein or fragment may then be further purified from the fusion protein by enzymatic cleavage of the fusion protein. Methods for preparing and using such fusion constructs for the purification of proteins are well known in the art and several kits are commercially available for this purpose. In light of the present disclosure, one is now enabled to employ such fusion constructs with the PS-interacting proteins.

3. Antibodies to the PS-interacting Proteins

The present invention also provides antibodies, and methods of making antibodies, which selectively bind to the PS-interacting proteins or fragments thereof. Of particular importance, by identifying the PS-interacting domains of the PS-interacting proteins, and methods of identifying mutant forms of the PS-interacting proteins associated with Alzheimer's Disease, the present invention provides antibodies, and methods of making antibodies, which will selectively bind to and, thereby, identify and/or distinguish normal and mutant (i.e., pathogenic) forms of the PS-interacting proteins. The antibodies of the invention have utility as laboratory reagents for, inter alia, immunoaffinity purification of the PS-interacting proteins, Western blotting to identify cells or tissues expressing the PS-interacting proteins, and immunocytochemistry or immunofluorescence techniques to establish the subcellular location of the proteins. In addition, as described below, the antibodies of the invention may be used as diagnostics tools to identify carriers of AD-related PS-interacting protein alleles, or as therapeutic tools to selectively bind and inhibit pathogenic forms of the PS-interacting proteins in vivo.

The antibodies of the invention may be generated using the entire PS-interacting proteins of the invention, or using any PS-interacting protein epitope which is characteristic of that protein and which substantially distinguishes it from other host proteins. Any method of choosing antigenic determinants known in the art may, of course, be employed. Such epitopes may be identified by comparing sequences of, for example, 4-10 amino acid residues from a PS-interacting protein sequence to computer databases of protein sequences from the relevant host. In addition, larger fragments (e.g., 8-20 or, preferably, 9-15 residues) including one or more potential epitopes may also be employed. Antibodies to the PS-interacting domains (identified by the yeast two-hybrid assays described below) are expected to have the greatest utility both diagnostically and therapeutically. On the other hand, antibodies against highly conserved domains are expected to have the greatest utility for purification or identification of PS-interacting proteins.

PS-interacting protein immunogen preparations may be produced from crude extracts (e.g., lysates or membrane fractions of cells highly expressing the proteins), from proteins or peptides substantially purified from cells which naturally or recombinantly express them or, for short immunogens, by chemical peptide synthesis. The immunogens may also be in the form of a fusion protein in which the non-PS-interacting protein region is chosen for its adjuvant properties. As used herein, a PS-interacting protein immunogen shall be defined as a preparation including a peptide comprising at least 4-8, and preferably at least 9-15 consecutive amino acid residues of a PS-interacting proteins, as disclosed or otherwise enabled herein. Sequences of fewer residues may, of course, also have utility depending upon the intended use and future technological developments. Therefore, any PS-interacting protein derived sequences which are employed to generate antibodies to the PS-interacting proteins should be regarded as PS-interacting protein immunogens.

The antibodies of the invention may be polyclonal or monoclonal, or may be antibody fragments, including Fab fragments, F(ab')₂, and single chain antibody fragments. In addition, after identifying useful antibodies by the method of the invention, recombinant antibodies may be generated, including any of the antibody

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fragments listed above, as well as humanized antibodies based upon non-human antibodies to the PS-interacting proteins. In light of the present disclosure, as well as the characterization of other PS-interacting proteins enabled herein, one of ordinary skill in the art may produce the above-described antibodies by any of a variety of standard means well known in the art. For an overview of antibody techniques, see 5 Antibody Engineering: A Practical Guide, Borrebaek, ed., W.H. Freeman & Company, NY (1992), or Antibody Engineering, 2nd Ed., Borrebaek, ed., Oxford University Press, Oxford (1995).

As a general matter, polyclonal antibodies may be generated by first 10 immunizing a mouse, rabbit, goat or other suitable animal with the PS-interacting protein immunogen in a suitable carrier. To increase the immunogenicity of the preparation, the immunogen may be coupled to a carrier protein or mixed with an adjuvant (e.g., Freund's adjuvant). Booster injections, although not necessary are recommended. After an appropriate period to allow for the development of a humoral 15 response, preferably several weeks, the animals may be bled and the sera may be purified to isolate the immunoglobulin component.

Similarly, as a general matter, monoclonal anti-PS-interacting protein antibodies may be produced by first injecting a mouse, rabbit, goat or other suitable animal with a PS-interacting protein immunogen in a suitable carrier. As above, 20 carrier proteins or adjuvants may be utilized and booster injections (e.g., bi- or tri-weekly over 8-10 weeks) are recommended. After allowing for development of a humoral response, the animals are sacrificed and their spleens are removed and resuspended in, for example, phosphate buffered saline (PBS). The spleen cells serve as a source of lymphocytes, some of which are producing antibody of the appropriate 25 specificity. These cells are then fused with an immortalized cell line (e.g., myeloma), and the products of the fusion are plated into a number of tissue culture wells in the presence of a selective agent such as HAT. The wells are serially screened and replated, each time selecting cells making useful antibody. Typically, several screening and replating procedures are carried out until over 90% of the wells contain 30 single clones which are positive for antibody production. Monoclonal antibodies

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produced by such clones may be purified by standard methods such as affinity chromatography using Protein A Sepharose, by ion-exchange chromatography, or by variations and combinations of these techniques.

The antibodies of the invention may be labelled or conjugated with other
5 compounds or materials for diagnostic and/or therapeutic uses. For example, they may be coupled to radionuclides, fluorescent compounds, or enzymes for imaging or therapy, or to liposomes for the targeting of compounds contained in the liposomes to a specific tissue location.

10 4. Transformed Cell Lines

The present invention also provides for cells or cell lines, both prokaryotic and eukaryotic, which have been transformed or transfected with the nucleic acids of the present invention so as to cause clonal propagation of those nucleic acids and/or
15 expression of the proteins or peptides encoded thereby. Such cells or cell lines will have utility both in the propagation and production of the nucleic acids and proteins of the present invention but also, as further described herein, as model systems for diagnostic and therapeutic assays. In particular, it is expected that cells co-transformed with PS-interacting protein sequences as well as presenilin sequences will have improved utility as models of the biochemical pathways which may be affected
20 in AD. For example, cells co-transformed with the interacting domains of PS-interacting sequences and presenilins in yeast two-hybrid fusion constructs, will have utility in screening for compounds which either enhance or inhibit interactions between these domains. Similarly, for cells transformed with a heterospecific presenilin, co-transformation with a similarly heterospecific PS-interacting protein, or
25 co-transformation and homologous recombination to introduce a similarly heterospecific PS-interacting domain of a PS-interacting protein (e.g., "humanizing" a non-human endogenous PS-interacting protein), will result in a better model system for studying the interactions of the presenilins and the PS-interacting proteins. Cells transformed with only PS-interacting sequences will, of course, have utility of their

own for studying the role of these proteins in the etiology of AD, and also as precursors for presenilin co-transformed cells.

As used herein, the term "transformed cell" is intended to embrace any cell, or the descendant of any cell, into which has been introduced any of the nucleic acids of the invention, whether by transformation, transfection, infection, or other means. Methods of producing appropriate vectors, transforming cells with those vectors, and identifying transformants are well known in the art and are only briefly reviewed here (see, for example, Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York).

Prokaryotic cells useful for producing the transformed cells of the invention include members of the bacterial genera Escherichia (e.g., E. coli), Pseudomonas (e.g., P. aeruginosa), and Bacillus (e.g., B. subtilis, B. stearothermophilus), as well as many others well known and frequently used in the art. Prokaryotic cells are particularly useful for the production of large quantities of the proteins or peptides of the invention (e.g., normal or mutant PS-interacting proteins, fragments of the PS-interacting proteins, fusion proteins of the PS-interacting proteins). Bacterial cells (e.g., E. coli) may be used with a variety of expression vector systems including, for example, plasmids with the T7 RNA polymerase/promoter system, bacteriophage λ regulatory sequences, or M13 Phage mGPI-2. Bacterial hosts may also be transformed with fusion protein vectors which create, for example, lacZ, trpE, maltose-binding protein, poly-His tags, or glutathione-S-transferase fusion proteins. All of these, as well as many other prokaryotic expression systems, are well known in the art and widely available commercially (e.g., pGEX-27 (Amrad, USA) for GST fusions).

Eukaryotic cells and cell lines useful for producing the transformed cells of the invention include mammalian cells and cell lines (e.g., PC12, COS, CHO, fibroblasts, myelomas, neuroblastomas, hybridomas, human embryonic kidney 293, oocytes, embryonic stem cells), insect cells lines (e.g., using baculovirus vectors such as pPbac or pMbac (Stratagene, La Jolla, CA)), yeast (e.g., using yeast expression

vectors such as pYESHIS (Invitrogen, CA)), and fungi. Eukaryotic cells are particularly useful for embodiments in which it is necessary that the PS-interacting proteins, or functional fragments thereof, perform the functions and/or undergo the intracellular interactions associated with either the normal or mutant proteins. Thus, for example, transformed eukaryotic cells are preferred for use as models of PS-interacting protein function or interaction, and assays for screening candidate therapeutics preferably employ transformed eukaryotic cells.

To accomplish expression in eukaryotic cells, a wide variety of vectors have been developed and are commercially available which allow inducible (e.g., LacSwitch expression vectors, Stratagene, La Jolla, CA) or cognate (e.g., pcDNA3 vectors, Invitrogen, Chatsworth, CA) expression of PS-interacting protein nucleotide sequences under the regulation of an artificial promoter element. Such promoter elements are often derived from CMV or SV40 viral genes, although other strong promoter elements which are active in eukaryotic cells can also be employed to induce transcription of PS-interacting protein nucleotide sequences. Typically, these vectors also contain an artificial polyadenylation sequence and 3' UTR which can also be derived from exogenous viral gene sequences or from other eukaryotic genes. Furthermore, in some constructs, artificial, non-coding, spliceable introns and exons are included in the vector to enhance expression of the nucleotide sequence of interest. These expression systems are commonly available from commercial sources and are typified by vectors such as pcDNA3 and pZeoSV (Invitrogen, San Diego, CA). Innumerable commercially-available as well as custom-designed expression vectors are available from commercial sources to allow expression of any desired PS-interacting protein transcript in more or less any desired cell type, either constitutively or after exposure to a certain exogenous stimulus (e.g., withdrawal of tetracycline or exposure to IPTG).

Vectors may be introduced into the recipient or "host" cells by various methods well known in the art including, but not limited to, calcium phosphate transfection, strontium phosphate transfection, DEAE dextran transfection, electroporation, lipofection (e.g., Dosper Liposomal transfection reagent, Boehringer

Mannheim, Germany), microinjection, ballistic insertion on micro-beads, protoplast fusion or, for viral or phage vectors, by infection with the recombinant virus or phage.

5. Transgenic Animal Models

5 The present invention also provides for the production of transgenic non-human animal models in which mutant or wild type PS-interacting protein sequences are expressed, or in which the PS-interacting protein genes have been inactivated (e.g., "knock-out" deletions), for the study of Alzheimer's Disease, for the screening of candidate pharmaceutical compounds, for the creation of explanted mammalian CNS
10 cell cultures (e.g., neuronal, glial, organotypic or mixed cell cultures), and for the evaluation of potential therapeutic interventions. Prior to the present invention, a partial animal model for Alzheimer's Disease existed via the insertion and over-expression of a mutant form of the human amyloid precursor protein gene as a minigene under the regulation of the platelet-derived growth factor β receptor
15 promoter element (Games et al., 1995). This mutant (β APP₇₁₇ Val \rightarrow Ile) causes the appearance of synaptic pathology and amyloid β peptide deposition in the brain of transgenic animals bearing this transgene in high copy number. These changes in the brain of the transgenic animal are very similar to that seen in human AD (Games et al., 1995). It is, however, as yet unclear whether these animals become demented, but
20 there is general consensus that it is now possible to recreate at least some aspects of AD in mice. In addition, transgenic animal models in which the presenilin genes are genetically engineered are disclosed in PCT Publication WO96/34099. These transgenic animal models have been shown to have altered A β production and altered hippocampus-dependent memory function.

25 Animal species suitable for use in the animal models of the present invention include, but are not limited to, rats, mice, hamsters, guinea pigs, rabbits, dogs, cats, goats, sheep, pigs, and non-human primates (e.g., Rhesus monkeys, chimpanzees). For initial studies, transgenic rodents (e.g., mice) may be preferred due to their relative ease of maintenance and shorter life spans. However, transgenic yeast
30 or invertebrates (e.g., nematodes, insects) may be preferred for some studies because

they will allow for even more rapid and inexpensive screening. For example, invertebrates bearing mutant PS-interacting protein homologues (or mammalian PS-interacting protein transgenes) which cause a rapidly occurring and easily scored phenotype (e.g., abnormal vulva or eye development after several days) can be used as
5 screens for drugs which block the effect of the mutant gene. Such invertebrates may prove far more rapid and efficient for mass screenings than larger vertebrate animals. Once lead compounds are found through such screens, they may be tested in higher animals such as rodents. Ultimately, transgenic non-human primates may be preferred for longer term studies due to their greater similarity to humans and their higher
10 cognitive abilities.

Using the nucleic acids disclosed and otherwise enabled herein, there are now several available approaches for the creation of a transgenic animal model for Alzheimer's Disease. Thus, the enabled animal models include: (1) Animals in which sequences encoding at least a functional domain of a normal human PS-interacting
15 protein gene have been recombinantly introduced into the genome of the animal as an additional gene, under the regulation of either an exogenous or an endogenous promoter element, and as either a minigene or a large genomic fragment; in which sequences encoding at least a functional domain of a normal human PS-interacting protein gene have been recombinantly substituted for one or both copies of the
20 animal's homologous PS-interacting protein gene by homologous recombination or gene targeting; and/or in which one or both copies of one of the animal's homologous PS-interacting protein genes have been recombinantly "humanized" by the partial substitution of sequences encoding the human homologue by homologous recombination or gene targeting. These animals are useful for evaluating the effects
25 of the transgenic procedures, and the effects of the introduction or substitution of a human or humanized PS-interacting protein gene. (2) Animals in which sequences encoding at least a functional domain of a mutant (i.e., pathogenic) human PS-interacting protein gene have been recombinantly introduced into the genome of the animal as an additional gene, under the regulation of either an exogenous or an
30 endogenous promoter element, and as either a minigene or a large genomic fragment;

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in which sequences encoding at least a functional domain of a mutant human PS-interacting protein gene have been recombinantly substituted for one or both copies of the animal's homologous PS-interacting protein gene by homologous recombination or gene targeting; and/or in which one or both copies of one of the animal's

5 homologous PS-interacting protein genes have been recombinantly "humanized" by the partial substitution of sequences encoding a mutant human homologue by homologous recombination or gene targeting. These animals are useful as models which will display some or all of the characteristics, whether at the biochemical, physiological and/or behavioral level, of humans carrying one or more alleles which

10 are pathogenic of Alzheimer's Disease or other diseases associated with mutations in the PS-interacting protein genes. (3) Animals in which sequences encoding at least a functional domain of a mutant version of one of that animal's PS-interacting protein genes (bearing, for example, a specific mutation corresponding to, or similar to, one of the pathogenic mutations of the human PS-interacting proteins) have been

15 recombinantly introduced into the genome of the animal as an additional gene, under the regulation of either an exogenous or an endogenous promoter element, and as either a minigene or a large genomic fragment; and/or in which sequences encoding at least a functional domain of a mutant version of one of that animal's PS-interacting protein genes (bearing, for example, a specific mutation corresponding to, or similar to, one of the pathogenic mutations of the human PS-interacting proteins) have been

20 recombinantly substituted for one or both copies of the animal's homologous PS-interacting protein gene by homologous recombination or gene targeting. These animals are also useful as models which will display some or all of the characteristics, whether at the biochemical, physiological and/or behavioral level, of humans carrying one or more alleles which are pathogenic of Alzheimer's Disease. (4) "Knock-out"

25 animals in which one or both copies of one of the animal's PS-interacting protein genes have been partially or completely deleted by homologous recombination or gene targeting, or have been inactivated by the insertion or substitution by homologous recombination or gene targeting of exogenous sequences (e.g., stop codons, lox p sites). Such animals are useful models to study the effects which loss of

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PS-interacting protein gene expression may have, to evaluate whether loss of function is preferable to continued expression of mutant forms, and to examine whether other genes can be recruited to replace a mutant PS-interacting protein or to intervene with the effects of other genes (e.g., PS1, PS2, APP or ApoE) causing AD as a treatment
5 for AD or other disorders. For example, a normal PS-interacting protein gene may be necessary for the action of mutant presenilin or APP genes to actually be expressed as AD and, therefore, transgenic PS-interacting protein animal models may be of use in elucidating such multigenic interactions.

In addition to transgenic animal models in which the expression of one or
10 more of the PS-interacting proteins is altered, the present invention also provides for the production of transgenic animal models in which the expression of one or more of the presenilins, APP, or ApoE is altered. The nucleic acids encoding the presenilins, APP, and ApoE are known in the art, a methods for producing transgenic animals with these sequences are also known (see, e.g., PCT Publication WO96/34099; Games et
15 al., 1995). Indeed, because non-human animals may differ from humans not only in their PS-interacting protein sequences, but also in the sequences of their presenilin, APP and/or ApoE homologues, it is particularly contemplated that transgenics may be produced which bear recombinant normal or mutant human sequences for at least one presenilin, APP and/or ApoE gene in addition to recombinant sequences for one or
20 more PS-interacting proteins. Such co-transformed animal models would possess more elements of the human molecular biology and, therefore, are expected to be better models of human disorders. Thus, in accordance with the present invention, transgenic animal models may be produced bearing normal or mutant sequences for one or more PS-interacting proteins, or interacting domains of these proteins. These
25 animals will have utility in that they can be crossed with animals bearing a variety of normal or mutant presenilin, APP or ApoE sequences to produce co-transformed animal models. Furthermore, as detailed below, it is expected that mutations in the PS-interacting genes, like mutations in the presenilins themselves, may be causative of Alzheimer's Disease and/or other disorders as well (e.g., other cognitive,
30 intellectual, neurological or psychological disorders such as cerebral hemorrhage.

schizophrenia, depression, mental retardation and epilepsy). Therefore, transgenic animal models bearing normal or mutant sequences corresponding to the PS-interacting proteins, absent transformation with any presenilin, APP or ApoE sequences, will have utility of their own in the study of such disorders.

5 As detailed below, preferred choices for transgenic animal models transformed with PS-interacting proteins, or domains of PS-interacting proteins, include those transformed with normal or mutant sequences corresponding to the clones identified and described in Example 1 and disclosed in SEQ ID NOs: 1-12. These clones, which interact with normal or mutant PS1 TM6→7 loop domains, were
10 identified according to the methods described in Example 1, below, and PCT Publication WO96/34099. These clones, longer nucleic acid sequences comprising these clones, and other clones identified according to this and other methods of the invention (e.g., allelic and splice variants or heterospecific homologues of these clones) may all be employed in accordance with the present invention to produce
15 animal models which, with or without co-transformation with presenilin, APP and/or ApoE sequences, will have utility in the study of Alzheimer's Disease and/or other cognitive, intellectual, neurological or psychological disorders.

Thus, using the nucleic acids disclosed and otherwise enabled herein, one of ordinary skill in the art may now produce any of the following types of transgenic
20 animal models with altered PS-interacting protein expression: (1) Animals in which sequences encoding at least a functional domain of a normal human PS-interacting protein gene have been recombinantly introduced into the genome of the animal as an additional gene, under the regulation of either an exogenous or an endogenous promoter element, and as either a minigene or a large genomic fragment; in which
25 sequences encoding at least a functional domain of a normal human PS-interacting protein gene have been recombinantly substituted for one or both copies of the animal's homologous PS-interacting protein gene by homologous recombination or gene targeting; and/or in which one or both copies of one of the animal's homologous PS-interacting protein genes have been recombinantly "humanized" by the partial
30 substitution of sequences encoding the human homologue by homologous

recombination or gene targeting. These animals are particularly useful for providing transgenic models which express human PS-interacting proteins as well as human presenilin proteins. They are also useful in evaluating the effects of the transgenic procedures, and the effects of the introduction or substitution of a human or

5 humanized PS-interacting protein gene. (2) Animals in which sequences encoding at least a functional domain of a mutant (i.e., pathogenic) human PS-interacting protein gene have been recombinantly introduced into the genome of the animal as an additional gene, under the regulation of either an exogenous or an endogenous promoter element, and as either a minigene or a large genomic fragment; in which

10 sequences encoding at least a functional domain of a mutant human PS-interacting protein gene have been recombinantly substituted for one or both copies of the animal's homologous PS-interacting protein gene by homologous recombination or gene targeting; and/or in which one or both copies of one of the animal's homologous PS-interacting protein genes have been recombinantly "humanized" by the partial

15 substitution of sequences encoding a mutant human homologue by homologous recombination or gene targeting. These animals are useful as models which will display some or all of the characteristics, whether at the biochemical, physiological and/or behavioral level, of humans carrying one or more alleles which are pathogenic of Alzheimer's Disease or other diseases associated with mutations in these PS-

20 interacting genes. (3) Animals in which sequences encoding at least a functional domain of a mutant version of one of that animal's PS-interacting protein genes (bearing, for example, a specific mutation corresponding to, or similar to, one of the pathogenic mutations of the human PS-interacting proteins) have been recombinantly introduced into the genome of the animal as an additional gene, under the regulation

25 of either an exogenous or an endogenous promoter element, and as either a minigene or a large genomic fragment; and/or in which sequences encoding at least a functional domain of a mutant version of one of that animal's PS-interacting protein genes (bearing, for example, a specific mutation corresponding to, or similar to, one of the pathogenic mutations of the humans PS-interacting proteins) have been recombinantly

30 substituted for one or both copies of the animal's homologous PS-interacting protein

gene by homologous recombination or gene targeting. These animals are also useful as models which will display some or all of the characteristics, whether at the biochemical, physiological and/or behavioral level, of humans carrying one or more alleles which are pathogenic of Alzheimer's Disease. (4) "Knock-out" animals in which one or both copies of one of the animal's PS-interacting protein genes have been partially or completely deleted by homologous recombination or gene targeting, or have been inactivated by the insertion or substitution by homologous recombination or gene targeting of exogenous sequences (e.g., stop codons, lox p sites). Such animals are useful models to study the effects which loss of PS-interacting protein gene expression may have, to evaluate whether loss of function is preferable to continued expression, and to examine whether other genes can be recruited to replace a mutant PS-interacting protein or to intervene with the effects of other genes (e.g., APP or ApoE) causing AD as a treatment for AD or other disorders. For example, a normal PS-interacting protein may be necessary for the action of mutant PS1, PS2 or APP genes to actually be expressed as AD and, therefore, transgenic PS-interacting protein animal models may be of use in elucidating such multigenic interactions.

In some preferred embodiments, transgenic animal models are produced in which just the PS-interacting domains of the PS-interacting proteins are introduced into the genome of the animal by homologous recombination. Thus, for example, preferred embodiments include transgenic animals in which the PS-interacting domains of PS-interacting proteins are "humanized" by homologous recombination with sequences from human PS-interacting proteins. These animals may then be bred with transgenics in which normal or mutant presenilin sequences have been introduced. The progeny of these animals, having both human presenilin and human PS-interacting protein sequences, will provide improved animal models for Alzheimer's Disease.

To create an animal model (e.g., a transgenic mouse), a normal or mutant PS-interacting gene (e.g., normal or mutant S5a, GT24, p0071, Rab11, etc.), or a normal or mutant version of a recombinant nucleic acid encoding at least a functional

domain of a PS-interacting gene (e.g., the PS-interacting domains obtained in the yeast two-hybrid system), can be inserted into a germ line or stem cell using standard techniques of oocyte microinjection, or transfection or microinjection into embryonic stem cells. Animals produced by these or similar processes are referred to as
5 transgenic. Similarly, if it is desired to inactivate or replace an endogenous presenilin or PS-interacting protein gene, homologous recombination using embryonic stem cells may be employed. Animals produced by these or similar processes are referred to as "knock-out" (inactivation) or "knock-in" (replacement) models.

For oocyte injection, one or more copies of the recombinant DNA
10 constructs of the present invention may be inserted into the pronucleus of a just-fertilized oocyte. This oocyte is then reimplanted into a pseudo-pregnant foster mother. The liveborn animals are screened for integrants using analysis of DNA (e.g., from the tail veins of offspring mice) for the presence of the inserted recombinant transgene sequences. The transgene may be either a complete genomic sequence
15 injected as a YAC, BAC, PAC or other chromosome DNA fragment, a cDNA with either the natural promoter or a heterologous promoter, or a minigene containing all of the coding region and other elements found to be necessary for optimum expression.

Retroviral infection of early embryos can also be done to insert the recombinant DNA constructs of the invention. In this method, the transgene (e.g., a
20 normal or mutant S5a, GT24, p0071, Rab 11, etc., sequence) is inserted into a retroviral vector which is used to infect embryos (e.g., mouse or non-human primate embryos) directly during the early stages of development to generate chimeras, some of which will lead to germline transmission.

Homologous recombination using stem cells allows for the screening of
25 gene transfer cells to identify the rare homologous recombination events. Once identified, these can be used to generate chimeras by injection of blastocysts, and a proportion of the resulting animals will show germline transmission from the recombinant line. This methodology is especially useful if inactivation of a gene is desired. For example, inactivation of the S5a gene in mice may be accomplished by
30 designing a DNA fragment which contains sequences from an S5a coding region

flanking a selectable marker. Homologous recombination leads to the insertion of the marker sequences in the middle of the coding region, causing inactivation of the S5a gene and/or deletion of internal sequences. DNA analysis of individual clones can then be used to recognize the homologous recombination events.

5 The techniques of generating transgenic animals, as well as the techniques for homologous recombination or gene targeting, are now widely accepted and practiced. A laboratory manual on the manipulation of the mouse embryo, for example, is available detailing standard laboratory techniques for the production of transgenic mice (Hogan et al., 1986). To create a transgene, the target sequence of
10 interest (e.g., normal or mutant presenilin sequences, normal or mutant PS-interacting protein sequences) are typically ligated into a cloning site located downstream of some promoter element which will regulate the expression of RNA from the sequence. Downstream of the coding sequence, there is typically an artificial polyadenylation sequence. In the transgenic models that have been used to
15 successfully create animals which mimic aspects of inherited human neurodegenerative diseases, the most successful promoter elements have been the platelet-derived growth factor receptor β gene subunit promoter and the hamster prion protein gene promoter, although other promoter elements which direct expression in central nervous system cells would also be useful. An alternate approach to creating a
20 transgene is to use an endogenous presenilin or PS-interacting protein gene promoter and regulatory sequences to drive expression of the transgene. Finally, it is possible to create transgenes using large genomic DNA fragments such as YACs which contain the entire desired gene as well as its appropriate regulatory sequences. Such constructs have been successfully used to drive human APP expression in transgenic
25 mice (Lamb et al., 1993).

Animal models can also be created by targeting the endogenous presenilin or PS-interacting protein gene in order to alter the endogenous sequence by homologous recombination. These targeting events can have the effect of removing endogenous sequence (knock-out) or altering the endogenous sequence to create an
30 amino acid change associated with human disease or an otherwise abnormal sequence

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(e.g., a sequence which is more like the human sequence than the original animal sequence) (knock-in animal models). A large number of vectors are available to accomplish this and appropriate sources of genomic DNA for mouse and other animal genomes to be targeted are commercially available from companies such as

5 GenomeSystems Inc. (St. Louis, Missouri, USA). The typical feature of these targeting vector constructs is that 2 to 4 kb of genomic DNA is ligated 5' to a selectable marker (e.g., a bacterial neomycin resistance gene under its own promoter element termed a "neomycin cassette"). A second DNA fragment from the gene of interest is then ligated downstream of the neomycin cassette but upstream of a second

10 selectable marker (e.g., thymidine kinase). The DNA fragments are chosen such that mutant sequences can be introduced into the germ line of the targeted animal by homologous replacement of the endogenous sequences by either one of the sequences included in the vector. Alternatively, the sequences can be chosen to cause deletion of sequences that would normally reside between the left and right arms of the vector

15 surrounding the neomycin cassette. The former is known as a knock-in, the latter is known as a knock-out. Again, innumerable model systems have been created, particularly for targeted knock-outs of genes including those relevant to neurodegenerative diseases (e.g., targeted deletions of the murine APP gene by Zheng et al., 1995; targeted deletion of the murine prion gene associated with adult onset

20 human CNS degeneration by Bueler et al., 1996).

Finally, equivalents of transgenic animals, including animals with mutated or inactivated presenilin genes, or mutated or inactivated PS-interacting protein genes, may be produced using chemical or X-ray mutagenesis of gametes, followed by fertilization. Using the isolated nucleic acids disclosed or otherwise enabled herein,

25 one of ordinary skill may more rapidly screen the resulting offspring by, for example, direct sequencing RFLP, PCR, or hybridization analysis to detect mutants, or Southern blotting to demonstrate loss of one allele by dosage.

6. Assays for Drugs Which Affect PS-Interacting Protein Expression

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In another series of embodiments, the present invention provides assays for identifying small molecules or other compounds which are capable of inducing or inhibiting the expression of the PS-interacting genes and proteins (e.g., S5a or GT24). The assays may be performed in vitro using non-transformed cells, immortalized cell
5 lines, or recombinant cell lines, or in vivo using the transgenic animal models enabled herein.

In particular, the assays may detect the presence of increased or decreased expression of S5a, GT24, p0071, Rab 11, or other PS-interacting genes or proteins on the basis of increased or decreased mRNA expression (using, e.g., the nucleic acid
10 probes disclosed and enabled herein), increased or decreased levels of PS-interacting proteins (using, e.g., the anti-PS-interacting protein antibodies disclosed and enabled herein), or increased or decreased levels of expression of a marker gene (e.g., β -galactosidase or luciferase) operably joined to a PS-interacting protein 5' regulatory region in a recombinant construct.

15 Thus, for example, one may culture cells known to express a particular PS-interacting protein and add to the culture medium one or more test compounds. After allowing a sufficient period of time (e.g., 0-72 hours) for the compound to induce or inhibit the expression of the PS-interacting protein, any change in levels of expression from an established baseline may be detected using any of the techniques described
20 above and well known in the art. In particularly preferred embodiments, the cells are from an immortalized cell line such as a human neuroblastoma, glioblastoma or a hybridoma cell line. Using the nucleic acid probes and /or antibodies disclosed and enabled herein, detection of changes in the expression of a PS-interacting protein, and thus identification of the compound as an inducer or repressor of PS-interacting
25 protein expression, requires only routine experimentation.

In particularly preferred embodiments, a recombinant assay is employed in which a reporter gene such a β -galactosidase, green fluorescent protein, alkaline phosphatase, or luciferase is operably joined to the 5' regulatory regions of a PS-interacting protein gene. Preferred vectors include the Green Lantern 1 vector
30 (GIBCO/BRL, Gaithersburg, MD) and the Great EScAPe pSEAP vector (Clontech,

Palo Alto). The PS-interacting protein regulatory regions may be easily isolated and cloned by one of ordinary skill in the art in light of the present disclosure of coding regions from these genes. The reporter gene and regulatory regions are joined in-frame (or in each of the three possible reading frames) so that transcription and translation of the reporter gene may proceed under the control of the PS-interacting protein regulatory elements. The recombinant construct may then be introduced into any appropriate cell type, although mammalian cells are preferred, and human cells are most preferred. The transformed cells may be grown in culture and, after establishing the baseline level of expression of the reporter gene, test compounds may be added to the medium. The ease of detection of the expression of the reporter gene provides for a rapid, high through-put assay for the identification of inducers and repressors of the PS-interacting protein gene.

Compounds identified by this method will have potential utility in modifying the expression of the PS-interacting protein genes in vivo. These compounds may be further tested in the animal models disclosed and enabled herein to identify those compounds having the most potent in vivo effects. In addition, as described herein with respect to small molecules having binding activity for PS-interacting proteins, these molecules may serve as "lead compounds" for the further development of pharmaceuticals by, for example, subjecting the compounds to sequential modifications, molecular modeling, and other routine procedures employed in rational drug design.

7. Identification of Compounds with PS-Interacting Protein Binding Capacity

In light of the present disclosure, one of ordinary skill in the art is enabled to practice new screening methodologies which will be useful in the identification of proteins and other compounds which bind to, or otherwise directly interact with, the PS-interacting proteins. The proteins and compounds will include endogenous cellular components, aside from the presenilins, which interact with the PS-interacting proteins in vivo and which, therefore, provide new targets for pharmaceutical and therapeutic interventions, as well as recombinant, synthetic and otherwise exogenous

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compounds which may have PS-interacting protein binding capacity and, therefore, may be candidates for pharmaceutical agents. Thus, in one series of embodiments, cell lysates or tissue homogenates (e.g., human brain homogenates, lymphocyte lysates) may be screened for proteins or other compounds which bind to one of the
5 normal or mutant PS-interacting proteins. Alternatively, any of a variety of exogenous compounds, both naturally occurring and/or synthetic (e.g., libraries of small molecules or peptides), may be screened for PS-interacting protein binding capacity. Small molecules are particular preferred in this context because they are more readily absorbed after oral administration, have fewer potential antigenic
10 determinants, and/or are more likely to cross the blood brain barrier than larger molecules such as nucleic acids or proteins. The methods of the present invention are particularly useful in that they may be used to identify molecules which selectively or preferentially bind to a mutant form of a PS-interacting protein (rather than a normal form) and, therefore, may have particular utility in treating cases of AD which arise
15 from mutations in the PS-interacting proteins.

Once identified by the methods described above, the candidate compounds may then be produced in quantities sufficient for pharmaceutical administration or testing (e.g., μg or mg or greater quantities), and formulated in a pharmaceutically acceptable carrier (see, e.g., Remington's Pharmaceutical Sciences, Gennaro, A., ed.,
20 Mack Pub., 1990). These candidate compounds may then be administered to the transformed cells of the invention, to the transgenic animal models of the invention, to cell lines derived from the animal models or from human patients, or to Alzheimer's patients. The animal models described and enabled herein are of particular utility in further testing candidate compounds which bind to normal or mutant PS-interacting
25 proteins for their therapeutic efficacy.

In addition, once identified by the methods described above, the candidate compounds may also serve as "lead compounds" in the design and development of new pharmaceuticals. For example, as is well known in the art, sequential modification of small molecules (e.g., amino acid residue replacement with peptides;
30 functional group replacement with peptide or non-peptide compounds) is a standard

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approach in the pharmaceutical industry for the development of new pharmaceuticals. Such development generally proceeds from a "lead compound" which is shown to have at least some of the activity (e.g., PS-interacting protein binding or blocking ability) of the desired pharmaceutical. In particular, when one or more compounds
5 having at least some activity of interest (e.g., modulation of PS-interacting protein activity) are identified, structural comparison of the molecules can greatly inform the skilled practitioner by suggesting portions of the lead compounds which should be conserved and portions which may be varied in the design of new candidate compounds. Thus, the present invention also provides a means of identifying lead
10 compounds which may be sequentially modified to produce new candidate compounds for use in the treatment of Alzheimer's Disease. These new compounds then may be tested both for binding to PS-interacting proteins and/or blocking PS-interacting protein activity, and for therapeutic efficacy (e.g., in the animal models described herein). This procedure may be iterated until compounds having the desired
15 therapeutic activity and/or efficacy are identified.

In each of the present series of embodiments, an assay is conducted to detect binding between a "PS-interacting protein component" and some other moiety. Of particular utility will be sequential assays in which compounds are tested for the ability to bind to only normal or only mutant forms of the PS-interacting domains of
20 PS-interacting proteins in the binding assays. Such compounds are expected to have the greatest therapeutic utilities, as described more fully below. The "PS-interacting protein component" in these assays may be a complete normal or mutant form of a PS-interacting protein (e.g., S5a, GT24, p0071, Rab 11, etc.) but need not be. Rather, particular functional domains of the PS-interacting proteins, particularly the PS-
25 interacting domains as described above, may be employed either as separate molecules or as part of a fusion protein. For example, to isolate proteins or compounds that interact with these functional domains, screening may be carried out using fusion constructs and/or synthetic peptides corresponding to these regions. Thus, for S5a, GST-fusion peptides may be made including sequences corresponding
30 approximately to amino acids 70-377 of SEQ ID NO: 2 (included in clones Y2H29

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and Y2H31, see Example 1), approximately to amino acids 206-377 of SEQ ID NO: 2 (which includes protein-protein interaction motifs, see Ferrell et al., 1996), or to any other S5a domain of interest. Similarly, for GT24, GST- or other fusion peptides may be produced including sequences corresponding approximately to amino acids 440-
5 815 of SEQ ID NO: 4 (including part of the armadillo repeat segment). Obviously, various combinations of fusion proteins and PS-interacting protein functional domains are possible and these are merely examples. In addition, the functional domains may be altered so as to aid in the assay by, for example, introducing into the functional domain a reactive group or amino acid residue (e.g., cysteine) which will facilitate
10 immobilization of the domain on a substrate (e.g., using sulfhydryl reactions). Thus, for example, the PS-interacting domain of S5a may be synthesized containing an additional C-terminal cysteine residue to facilitate immobilization of the domain. Such peptides may be used to create an affinity substrate for affinity chromatography (Sulfo-link; Pierce) to isolate binding proteins for microsequencing. Similarly, other
15 functional domain or antigenic fragments may be created with modified residues (see, e.g., Example 4).

The proteins or other compounds identified by these methods may be purified and characterized by any of the standard methods known in the art. Proteins may, for example, be purified and separated using electrophoretic (e.g., SDS-PAGE,
20 2D PAGE) or chromatographic (e.g., HPLC) techniques and may then be microsequenced. For proteins with a blocked N-terminus, cleavage (e.g., by CNBr and/or trypsin) of the particular binding protein is used to release peptide fragments. Further purification/characterization by HPLC and microsequencing and/or mass spectrometry by conventional methods provides internal sequence data on such
25 blocked proteins. For non-protein compounds, standard organic chemical analysis techniques (e.g., IR, NMR and mass spectrometry; functional group analysis; X-ray crystallography) may be employed to determine their structure and identity.

Methods for screening cellular lysates, tissue homogenates, or small molecule libraries for candidate PS-interaction protein-binding molecules are well
30 known in the art and, in light of the present disclosure, may now be employed to

identify compounds which bind to normal or mutant PS-interacting protein components or which modulate PS-interacting protein activity as defined by non-specific measures (e.g., changes in intracellular Ca^{2+} , GTP/GDP ratio) or by specific measures (e.g., changes in A β peptide production or changes in the expression of other downstream genes which can be monitored by differential display, 2D gel electrophoresis, differential hybridization, or SAGE methods). The preferred methods involve variations on the following techniques: (1) direct extraction by affinity chromatography; (2) co-isolation of PS-interacting protein components and bound proteins or other compounds by immunoprecipitation; (3) the Biomolecular Interaction Assay (BIAcore); and (4) the yeast two-hybrid systems. These and others are discussed separately below.

A. Affinity Chromatography

In light of the present disclosure, a variety of affinity binding techniques well known in the art may be employed to isolate proteins or other compounds which bind to the PS-interacting protein disclosed or otherwise enabled herein. In general, a PS-interacting protein component may be immobilized on a substrate (e.g., a column or filter) and a solution including the test compound(s) is contacted with the PS-interacting protein, fusion or fragment under conditions which are permissive for binding. The substrate is then washed with a solution to remove unbound or weakly bound molecules. A second wash may then elute those compounds which strongly bound to the immobilized normal or mutant PS-interacting protein component. Alternatively, the test compounds may be immobilized and a solution containing one or more PS-interacting protein components may be contacted with the column, filter or other substrate. The ability of the PS-interacting protein component to bind to the test compounds may be determined as above or a labeled form of the PS-interacting protein component (e.g., a radio-labeled or chemiluminescent functional domain) may be used to more rapidly assess binding to the substrate-immobilized compound(s).

B. Co-Immunoprecipitation

Another well characterized technique for the isolation of PS-interacting protein components and their associated proteins or other compounds is direct

immunoprecipitation with antibodies. This procedure has been successfully used, for example, to isolate many of the synaptic vesicle associated proteins (Phizicky and Fields, 1994). Thus, either normal or mutant, free or membrane-bound PS-interacting protein components may be mixed in a solution with the candidate compound(s) under conditions which are permissive for binding, and the PS-interacting protein component may be immunoprecipitated. Proteins or other compounds which co-immunoprecipitate with the PS-interacting protein component may then be identified by standard techniques as described above. General techniques for immunoprecipitation may be found in, for example, Harlow and Lane, (1988)

10 Antibodies: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY.

The antibodies employed in this assay, as described and enabled herein, may be polyclonal or monoclonal, and include the various antibody fragments (e.g., Fab, F(ab')₂,) as well as single chain antibodies, and the like.

15 C. The Biomolecular Interaction Assay

Another useful method for the detection and isolation of binding proteins is the Biomolecular Interaction Assay or "BIAcore" system developed by Pharmacia Biosensor and described in the manufacturer's protocol (LKB Pharmacia, Sweden). In light of the present disclosure, one of ordinary skill in the art is now enabled to

20 employ this system, or a substantial equivalent, to identify proteins or other compounds having PS-interacting protein binding capacity. The BIAcore system uses an affinity purified anti-GST antibody to immobilize GST-fusion proteins onto a sensor chip. Obviously, other fusion proteins and corresponding antibodies may be substituted. The sensor utilizes surface plasmon resonance which is an optical

25 phenomenon that detects changes in refractive indices. A homogenate of a tissue of interest is passed over the immobilized fusion protein and protein-protein interactions are registered as changes in the refractive index. This system can be used to determine the kinetics of binding and to assess whether any observed binding is of physiological relevance.

D. The Yeast Two-Hybrid System

The yeast "two-hybrid" system takes advantage of transcriptional factors that are composed of two physically separable, functional domains (Phizicky and Fields, 1994). The most commonly used is the yeast GAL4 transcriptional activator consisting of a DNA binding domain and a transcriptional activation domain. Two different cloning vectors are used to generate separate fusions of the GAL4 domains to genes encoding potential binding proteins. The fusion proteins are co-expressed, targeted to the nucleus and, if interactions occur, activation of a reporter gene (e.g., lacZ) produces a detectable phenotype. For example, the Clontech Matchmaker System-2 may be used with the Clontech brain cDNA GAL4 activation domain fusion library with PS-interacting protein-GAL4 binding domain fusion clones (Clontech, Palo Alto, CA). In light of the disclosures herein, one of ordinary skill in the art is now enabled to produce a variety of PS-interacting protein fusions, including fusions including either normal or mutant functional domains of the PS-interacting proteins, and to screen such fusion libraries in order to identify PS-interacting protein binding proteins.

E. Other Methods

The nucleotide sequences and protein products, including both mutant and normal forms of these nucleic acids and their corresponding proteins, can be used with the above techniques to isolate other interacting proteins, and to identify other genes whose expression is altered by the over-expression of normal PS-interacting protein sequences, by the under-expression of normal PS-interacting protein sequences, or by the expression of mutant PS-interacting protein sequences. Identification of these other interacting proteins, as well as the identification of other genes whose expression levels are altered in AD will identify other gene targets which have direct relevance to the pathogenesis of this disease in its clinical or pathological forms. Specifically, other genes will be identified which may themselves be the site of other mutations causing Alzheimer's Disease, or which can themselves be targeted therapeutically (e.g., to reduce their expression levels to normal, or to pharmacologically block the effects of their over-expression) as a potential treatment

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for this disease. Specifically, these techniques rely on PCR-based and/or hybridization-based methods to identify genes which are differentially expressed between two conditions (a cell line expressing normal PS-interacting proteins compared to the same cell type expressing a mutant PS-interacting protein). These techniques include differential display, serial analysis of gene expression (SAGE), and mass-spectrometry of protein 2D-gels and subtractive hybridization (reviewed in Nowak, 1995 and Kahn, 1995).

As will be obvious to one of ordinary skill in the art, there are numerous other methods of screening individual proteins or other compounds, as well as large libraries of proteins or other compounds (e.g., phage display libraries and cloning systems from Stratagene, La Jolla, CA) to identify molecules which bind to normal or mutant PS-interacting protein components. All of these methods comprise the step of mixing a normal or mutant PS-interacting protein, fusion, or fragment with test compounds, allowing for binding (if any), and assaying for bound complexes. All such methods are now enabled by the present disclosure of substantially pure PS-interacting proteins, substantially pure PS-interacting functional domain fragments, PS-interacting protein fusion proteins, PS-interacting protein antibodies, and methods of making and using the same.

8. Disrupting PS-Interacting Protein Interactions

The ability to disrupt specific interactions of the PS-interacting proteins with the presenilins, or with other proteins, is potentially of great therapeutic value, and will be important in understanding the etiology of AD and in identifying additional targets for therapy. The methods used to identify compounds which disrupt PS-interacting protein interactions may be applied equally well to interactions involving either normal or mutant PS-interacting proteins.

Assays for compounds which can disrupt PS-interacting protein interactions may be performed by any of a variety of methods well known in the art. In essence, such assays will parallel those assays for identifying proteins and compounds with binding activity toward the PS-interacting proteins. Thus, once a

compound with binding activity for a PS-interacting protein is identified by any method, that method or an equivalent method may be performed in the presence of candidate compounds to identify compounds which disrupt the interaction. Thus, for example, the assay may employ methods including (1) affinity chromatography; (2) immunoprecipitation; (3) the Biomolecular Interaction Assay (BIAcore); or (4) the yeast two-hybrid systems. Such assays can be developed using either normal or mutant purified PS-interacting proteins, and/or either normal or mutant purified binding proteins (e.g., normal or mutant presenilins).

For affinity methods, either the PS-interacting protein or its binding partner may be affixed to a matrix, for example in a column, and the counterpart protein (e.g., the PS-interacting protein if presenilin or another binding partner is affixed to the matrix; or a presenilin or other binding partner if the PS-interacting protein is affixed to the matrix) is then exposed to the affixed protein/compound either before or after adding the candidate compound(s). In the absence of a disruptive effect by the candidate compound(s), the interaction between the PS-interacting protein and its binding partner will cause the counterpart protein to bind to the affixed protein. Any compound which disrupts the interaction will cause release of the counterpart protein from the matrix. Release of the counterpart protein from the matrix can be measured using methods known in the art.

For PS-interacting protein interactions which are detectable by yeast two-hybrid systems, these assays may also be employed to identify compounds which disrupt the interaction. Briefly, a PS-interacting protein and its binding partner (or appropriate structural domains of each) are employed in the fusion proteins of the system, and the cells are exposed to candidate compounds to determine their effect upon the expression of the reporter gene. By appropriate choice of a reporter gene, such a system can be readily adapted for high through-put screening of large libraries of compounds by, for example, using a reporter gene which confers resistance to an antibiotic which is present in the medium, or which rescues an auxotrophic strain grown in minimal medium.

These assays may be used to screen many different types of compounds for their disruptive effect on the interactions of the PS-interacting proteins. For example, the compounds may belong to a library of synthetic molecules, or be specifically designed to disrupt the interaction. The compounds may also be peptides

- 5 corresponding to the interacting domain of either protein. This type of assay can be used to identify compounds that disrupt a specific interaction between a given PS-interacting protein variant and a given binding partner. In addition, compounds that disrupt all interactions with PS-interacting proteins may be identified. For example, a compound that specifically disrupts the folding of PS-interacting proteins would be
- 10 expected to disrupt all interactions between PS-interacting proteins and other proteins. Alternatively, this type of disruption assay can be used to identify compounds which disrupt only a range of different PS-interacting protein interactions, or only a single PS-interacting protein interaction.

15 9. Methods of Identifying Compounds Modulating PS-Interacting Protein Activity

- In another series of embodiments, the present invention provides for methods of identifying compounds with the ability to modulate the activity of normal and mutant PS-interacting proteins. As used with respect to this series of
- embodiments, the term "activity" broadly includes gene and protein expression, PS-
- 20 interacting protein post-translation processing, trafficking and localization, and any functional activity (e.g., enzymatic, receptor-effector, binding, channel), as well as downstream affects of any of these. It is known that Alzheimer's Disease is associated with increased production of the long form of A β peptides, the appearance of amyloid plaques and neurofibrillary tangles, decreases in cognitive function, and apoptotic cell
- 25 death. Therefore, using the transformed cells and transgenic animal models of the present invention, cells obtained from subjects bearing normal or mutant PS-interacting protein genes, or animals or human subjects bearing naturally occurring normal or mutant PS-interacting proteins, it is now possible to screen candidate pharmaceuticals and treatments for their therapeutic effects by detecting changes in

one or more of these functional characteristics or phenotypic manifestations of normal or mutant PS-interacting protein expression.

Thus, the present invention provides methods for screening or assaying for proteins, small molecules or other compounds which modulate PS-interacting protein activity by contacting a cell in vivo or in vitro with a candidate compound and assaying for a change in a marker associated with normal or mutant PS-interacting protein activity. The marker associated with PS-interacting protein activity may be any measurable biochemical, physiological, histological and/or behavioral characteristic associated with PS-interacting protein expression. In particular, useful markers will include any measurable biochemical, physiological, histological and/or behavioral characteristic which distinguishes cells, tissues, animals or individuals bearing at least one mutant presenilin or PS-interacting protein gene from their normal counterparts. In addition, the marker may be any specific or non-specific measure of presenilin or PS-interacting protein activity. PS-interacting protein specific measures include measures of PS-interacting protein expression (e.g., PS-interacting protein mRNA or protein levels) which may employ the nucleic acid probes or antibodies of the present invention. Non-specific measures include changes in cell physiology such as pH, intracellular calcium, cyclic AMP levels, GTP/GDP ratios, phosphatidylinositol activity, protein phosphorylation, etc., which can be monitored on devices such as the cytosensor microphysiometer (Molecular Devices Inc., United States). The activation or inhibition of PS-interacting protein activity in its mutant or normal form can also be monitored by examining changes in the expression of other genes (e.g., the presenilins) which are specific to the PS-interacting protein pathway leading to Alzheimer's Disease. These can be assayed by such techniques as differential display, differential hybridization, and SAGE (sequential analysis of gene expression), as well as by two dimensional gel electrophoresis of cellular lysates. In each case, the differentially-expressed genes can be ascertained by inspection of identical studies before and after application of the candidate compound. Furthermore, as noted elsewhere, the particular genes whose expression is modulated by the administration of the candidate compound can be ascertained by cloning.

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nucleotide sequencing, amino acid sequencing, or mass spectrometry (reviewed in Nowak, 1995).

In general, a cell may be contacted with a candidate compound and, after an appropriate period (e.g., 0-72 hours for most biochemical measures of cultured
5 cells), the marker of presenilin or PS-interacting protein activity may be assayed and compared to a baseline measurement. The baseline measurement may be made prior to contacting the cell with the candidate compound or may be an external baseline established by other experiments or known in the art. The cell may be a transformed cell of the present invention or an explant from an animal or individual. In particular,
10 the cell may be an explant from a carrier of a presenilin or PS-interacting protein mutation (e.g., a human subject with Alzheimer's Disease) or an animal model of the invention (e.g., a transgenic nematode or mouse bearing a mutant presenilin or PS-interacting protein gene). To augment the effect of presenilin or PS-interacting protein mutations on the A β pathway, transgenic cells or animals may be employed
15 which have increased A β production. Preferred cells include those from neurological tissues such as neuronal, glial or mixed cell cultures; and cultured fibroblasts, liver, kidney, spleen, or bone marrow. The cells may be contacted with the candidate compounds in a culture in vitro or may be administered in vivo to a live animal or human subject. For live animals or human subjects, the test compound may be
20 administered orally or by any parenteral route suitable to the compound. For clinical trials of human subjects, measurements may be conducted periodically (e.g., daily, weekly or monthly) for several months or years.

Because most individuals bearing a mutation in a particular gene are heterozygous at that locus (i.e., bearing one normal and one mutant allele),
25 compounds may be tested for their ability to modulate normal as well as mutant presenilin or PS-interacting protein activity. Thus, for example, compounds which enhance the function of normal presenilins or PS-interacting proteins may have utility in treating Alzheimer's Disease or related disorders. Alternatively, because suppression of the activity of both normal and mutant copies of a gene in a
30 heterozygous individual may have less severe clinical consequences than progression

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of the associated disease, it may be desired to identify compound which inactivate or suppress all forms of the presenilins, the PS-interacting proteins, or their interactions. Preferably, however, compounds are identified which selectively or specifically inactivate or suppress the activity of mutant presenilin or PS-interacting proteins
5 without disrupting the function of their normal counterparts.

In light of the identification, characterization, and disclosure herein of a novel group of PS-interacting genes and proteins, the PS-interacting protein nucleic acid probes and antibodies, and the PS-interacting protein transformed cells and transgenic animals of the invention, one of ordinary skill in the art is now enabled by
10 perform a great variety of assays which will detect the modulation of presenilin and/or PS-interacting protein activity by candidate compounds. Particularly preferred and contemplated embodiments are discussed in some detail below.

A. PS-Interacting Protein Expression

In one series of embodiments, specific measures of PS-interacting protein
15 expression are employed to screen candidate compounds for their ability to affect presenilin activity. Thus, using the PS-interacting protein nucleic acids and antibodies disclosed and otherwise enabled herein, one may use mRNA levels or protein levels as a marker for the ability of a candidate compound to modulate PS-interacting protein activity. The use of such probes and antibodies to measure gene and protein
20 expression is well known in the art and discussed elsewhere herein. Of particular interest may be the identification of compounds which can alter the relative levels of different variants (e.g., mutant and normal) of the PS-interacting proteins.

B. Intracellular Localization

In another series of embodiments, compounds may be screened for their
25 ability to modulate the activity of the PS-interacting proteins based upon their effects on the trafficking and intracellular localization of the PS-interacting proteins. The presenilins and some of the PS-interacting proteins (e.g., S5a) have been seen immunocytochemically to be localized in membrane structures associated with the endoplasmic reticulum and Golgi apparatus. Differences in localization of mutant and
30 normal presenilins or PS-interacting proteins may, therefore, contribute to the etiology

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of Alzheimer's Disease and related disorders. Compounds which can affect the localization of these proteins may, therefore, be identified as potential therapeutics. Standard techniques known in the art may be employed to detect the localization of the presenilins and PS-interacting proteins. Generally, these techniques will employ the antibodies of the present invention, and in particular antibodies which selectively bind to one or more mutant PS-interacting proteins but not to normal proteins. As is well known in the art, such antibodies may be labeled by any of a variety of techniques (e.g., fluorescent or radioactive tags, labeled secondary antibodies, avidin-biotin, etc.) to aid in visualizing the intracellular location of these proteins. The PS-interacting proteins may be co-localized to particular structures, as is known in the art, using antibodies to markers of those structures (e.g., TGN38 for the Golgi, transferrin receptor for post-Golgi transport vesicles, LAMP2 for lysosomes). Western blots of purified fractions from cell lysates enriched for different intracellular membrane bound organelles (e.g., lysosomes, synaptosomes, Golgi) may also be employed.

B. Ion Regulation/Metabolism

In another series of embodiments, compounds may be screened for their ability to modulate the activity of the presenilins or PS-interacting proteins based upon measures in intracellular Ca^{2+} , Na^+ or K^+ levels or metabolism. As noted above, the presenilins are membrane associated proteins which may serve as, or interact with, ion receptors or ion channels. Thus, compounds may be screened for their ability to modulate presenilin and PS-interacting protein-related metabolism of calcium or other ions either in vivo or in vitro by, for example, measurements of ion channel fluxes and/or transmembrane voltage and/or current fluxes, using patch clamps, voltage clamps or fluorescent dyes sensitive to intracellular ion levels or transmembrane voltage. Ion channel or receptor function can also be assayed by measurements of activation of second messengers such as cyclic AMP, cGMP tyrosine kinases, phosphates, increases in intracellular Ca^{2+} levels, etc. Recombinantly made proteins may also be reconstructed in artificial membrane systems to study ion channel conductance and, therefore, the "cell" employed in such assays may comprise an

artificial membrane or cell. Assays for changes in ion regulation or metabolism can be performed on cultured cells expressing endogenous normal or mutant presenilins and PS-interacting proteins. Such studies also can be performed on cells transfected with vectors capable of expressing one of the presenilins or PS-interacting proteins, or
5 functional domains of one of the presenilins or PS-interacting proteins, in normal or mutant form. In addition, to enhance the signal measured in such assays, cells may be co-transfected with genes encoding ion channel proteins. For example, Xenopus oocytes or rat kidney (HEK293) cells may be co-transfected with sequences encoding rat brain Na⁺ β 1 subunits, rabbit skeletal muscle Ca²⁺ β 1 subunits, or rat heart K⁺ β 1
10 subunits. Changes in presenilin or PS-interacting protein-mediated ion channel activity can be measured by, for example, two-microelectrode voltage-clamp recordings in oocytes, by whole-cell patch-clamp recordings in HEK293 cells, or by equivalent means.

C. Apoptosis or Cell Death

15 In another series of embodiments, compounds may be screened for their ability to modulate the activity of the presenilins or PS-interacting proteins based upon their effects on presenilin or PS-interacting protein-related apoptosis or cell death. Thus, for example, baseline rates of apoptosis or cell death may be established for cells in culture, or the baseline degree of neuronal loss at a particular age may be
20 established post-mortem for animal models or human subjects, and the ability of a candidate compound to suppress or inhibit apoptosis or cell death may be measured. Cell death may be measured by standard microscopic techniques (e.g., light microscopy) or apoptosis may be measured more specifically by characteristic nuclear morphologies or DNA fragmentation patterns which create nucleosomal ladders (see,
25 e.g., Gavrieli et al., 1992; Jacobson et al., 1993; Vito et al., 1996). TUNEL may also be employed to evaluate cell death in brain (see, e.g., Lassmann et al., 1995). In preferred embodiments, compounds are screened for their ability to suppress or inhibit neuronal loss in the transgenic animal models of the invention. Transgenic mice bearing, for example, a mutant human, mutant mouse, or humanized mutant presenilin
30 or PS-interacting protein gene may be employed to identify or evaluate compounds

which may delay or arrest the neurodegeneration associated with Alzheimer's Disease. A similar transgenic mouse model, bearing a mutant APP gene, has recently been reported by Games et al. (1995).

D. A β Peptide Production

5 In another series of embodiments, compounds may be screened for their ability to modulate presenilin or PS-interacting protein-related changes in APP processing. The A β peptide is produced in several isoforms resulting from differences in APP processing. The A β peptide is a 39 to 43 amino acid derivative of β APP which is progressively deposited in diffuse and senile plaques and in blood vessels of
10 subjects with AD. In human brain, A β peptides are heterogeneous at both the N- and C-termini. Several observations, however, suggest that both the full length and N-terminal truncated forms of the long-tailed A β peptides ending at residue 42 or 43 (i.e., A β 1-42/43 and A β x-42/43) have a more important role in AD than do peptides ending at residue 40. Thus, A β 1-42/43 and A β x-42/43 are an early and prominent
15 feature of both senile plaques and diffuse plaques, while peptides ending at residue 40 (i.e., A β 1-40 and A β x-40) are predominantly associated with a subset of mature plaques and with amyloidotic blood vessels (see, e.g., Iwatsubo et al., 1995; Gravina et al., 1995; Tamaoka et al., 1995; Podlisny et al. 1995). Furthermore, the long-tailed isoforms have a greater propensity to fibril formation, and are thought to be more
20 neurotoxic than A β 1-40 peptides (Pike et al., 1993; Hilbich et al., 1991). Finally, missense mutations at codon 717 of the β APP gene are associated with early onset FAD, and result in overproduction of long-tailed A β in the brain of affected mutation carriers, in peripheral cells and plasma of both affected and presymptomatic carriers, and in cell lines transfected with β APP₇₁₇ mutant cDNAs (Tamaoka et al., 1994;
25 Suzuki et al., 1994).

Thus, in one series of embodiments, the present invention provides methods for screening candidate compounds for their ability to block or inhibit the increased production of long isoforms of the A β peptides in cells or transgenic animals expressing a normal or mutant presenilin gene and/or a normal or mutant PS-
30 interacting protein gene. In particular, the present invention provides such methods in

which cultured mammalian cells, such as brain cells or fibroblasts, have been transformed according to the methods disclosed herein, or in which transgenic animals, such as rodents or non-human primates, have been produced by the methods disclosed herein, to express relatively high levels of a normal or mutant presenilin or PS-interacting protein. Optionally, such cells or transgenic animals may also be transformed so as to express a normal or mutant form of the β APP protein at relatively high levels.

In this series of embodiments, the candidate compound is administered to the cell line or transgenic animals (e.g., by addition to the media of cells in culture; or by oral or parenteral administration to an animal) and, after an appropriate period (e.g., 0-72 hours for cells in culture, days or months for animal models), a biological sample is collected (e.g., cell culture supernatant or cell lysate from cells in culture; tissue homogenate or plasma from an animal) and tested for the level of the long isoforms of the $A\beta$ peptides. The levels of the peptides may be determined in an absolute sense (e.g., nMol/ml) or in a relative sense (e.g., ratio of long to short $A\beta$ isoforms). The $A\beta$ isoforms may be detected by any means known in the art (e.g., electrophoretic separation and sequencing) but, preferably, antibodies which are specific to the long isoform are employed to determine the absolute or relative levels of the $A\beta$ 1-42/43 or $A\beta$ x-42/43 peptides. Candidate pharmaceuticals or therapies which reduce the absolute or relative levels of these long $A\beta$ isoforms, particularly in the transgenic animal models of the invention, are likely to have therapeutic utility in the treatment of Alzheimer's Disease, or other disorders caused by mutations in the presenilins or PS-interacting proteins, or by other aberrations in APP metabolism.

E. Phosphorylation of Microtubule Associated Proteins

In another series of embodiments, candidate compounds may be screened for their ability to modulate presenilin or PS-interacting protein activity by assessing the effect of the compound on levels of phosphorylation of microtubule associated proteins (MAPs) such as tau. The abnormal phosphorylation of tau and other MAPs in the brains of victims of Alzheimer's Disease is well known in the art. Thus, compounds which prevent or inhibit the abnormal phosphorylation of MAPs may

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have utility in treating presenilin or PS-interacting protein-associated diseases such as AD. As above, cells from normal or mutant animals or subjects, or the transformed cell lines and animal models of the invention may be employed. Preferred assays will employ cell lines or animal models transformed with a mutant human or humanized mutant presenilin or PS-interacting protein gene. The baseline phosphorylation state of MAPs in these cells may be established and then candidate compounds may be tested for their ability to prevent, inhibit or counteract the hyperphosphorylation associated with mutants. The phosphorylation state of the MAPs may be determined by any standard method known in the art but, preferably, antibodies which bind selectively to phosphorylated or unphosphorylated epitopes are employed. Such antibodies to phosphorylation epitopes of the tau protein are known in the art (e.g., ALZ50).

10. Screening and Diagnostics for Alzheimer's Disease

15 A. General Diagnostic Methods

The PS-interacting genes and gene products, as well as the PS-interacting protein derived probes, primers and antibodies, disclosed or otherwise enabled herein, are useful in the screening for carriers of alleles associated with Alzheimer's Disease, for diagnosis of victims of Alzheimer's Disease, and for the screening and diagnosis of related presenile and senile dementias, psychiatric diseases such as schizophrenia and depression, and neurologic diseases such as stroke and cerebral hemorrhage, all of which are seen to a greater or lesser extent in symptomatic human subjects bearing mutations in the PS1 or PS2 genes or in the APP gene. Individuals at risk for Alzheimer's Disease, such as those with AD present in the family pedigree, or individuals not previously known to be at risk, may be routinely screened using probes to detect the presence of a mutant PS-interacting protein gene or protein by a variety of techniques. Diagnosis of inherited cases of these diseases can be accomplished by methods based upon the nucleic acids (including genomic and mRNA/cDNA sequences), proteins, and/or antibodies disclosed and enabled herein, including functional assays designed to detect failure or augmentation of the normal

presenilin or PS-interacting protein activity and/or the presence of specific new activities conferred by mutant PS-interacting proteins. Preferably, the methods and products are based upon the human nucleic acids, proteins or antibodies, as disclosed or otherwise enabled herein. As will be obvious to one of ordinary skill in the art, however, the significant evolutionary conservation of large portions of nucleotide and amino acid sequences, even in species as diverse as humans, mice, C. elegans, and Drosophila, allow the skilled artisan to make use of non-human homologues of the PS-interacting proteins to produce useful nucleic acids, proteins and antibodies, even for applications directed toward human or other animal subjects. Thus, for brevity of exposition, but without limiting the scope of the invention, the following description will focus upon uses of the human homologues of PS-interacting proteins and genes. It will be understood, however, that homologous sequences from other species will be equivalent for many purposes.

As will be appreciated by one of ordinary skill in the art, the choice of diagnostic methods of the present invention will be influenced by the nature of the available biological samples to be tested and the nature of the information required. Alzheimer's Disease is, of course, primarily a disease of the brain, but brain biopsies are invasive and expensive procedures, particularly for routine screening. Other tissues which express the presenilins or PS-interacting proteins at significant levels may, therefore, be preferred as sources for samples.

B. Protein Based Screens and Diagnostics

When a diagnostic assay is to be based upon PS-interacting proteins, a variety of approaches are possible. For example, diagnosis can be achieved by monitoring differences in the electrophoretic mobility of normal and mutant proteins. Such an approach will be particularly useful in identifying mutants in which charge substitutions are present, or in which insertions, deletions or substitutions have resulted in a significant change in the electrophoretic migration of the resultant protein. Alternatively, diagnosis may be based upon differences in the proteolytic cleavage patterns of normal and mutant proteins, differences in molar ratios of the

various amino acid residues, or by functional assays demonstrating altered function of the gene products.

In preferred embodiments, protein-based diagnostics will employ differences in the ability of antibodies to bind to normal and mutant PS-interacting proteins. Such diagnostic tests may employ antibodies which bind to the normal proteins but not to mutant proteins, or vice versa. In particular, an assay in which a plurality of monoclonal antibodies, each capable of binding to a mutant epitope, may be employed. The levels of anti-mutant antibody binding in a sample obtained from a test subject (visualized by, for example, radiolabelling, ELISA or chemiluminescence) may be compared to the levels of binding to a control sample. Alternatively, antibodies which bind to normal but not mutant proteins may be employed, and decreases in the level of antibody binding may be used to distinguish homozygous normal individuals from mutant heterozygotes or homozygotes. Such antibody diagnostics may be used for in situ immunohistochemistry using biopsy samples of CNS tissues obtained antemortem or postmortem, including neuropathological structures associated with these diseases such as neurofibrillary tangles and amyloid plaques, or may be used with fluid samples such as cerebrospinal fluid or with peripheral tissues such as white blood cells.

C. Nucleic Acid Based Screens and Diagnostics

When the diagnostic assay is to be based upon nucleic acids from a sample, the assay may be based upon mRNA, cDNA or genomic DNA. When mRNA is used from a sample, there are considerations with respect to source tissues and the possibility of alternative splicing. That is, there may be little or no expression of transcripts unless appropriate tissue sources are chosen or available, and alternative splicing may result in the loss of some information or difficulty in interpretation. Whether mRNA, cDNA or genomic DNA is assayed, standard methods well known in the art may be used to detect the presence of a particular sequence either in situ or in vitro (see, e.g., Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Press, Cold Spring Harbor, NY). As a general matter, however, any tissue with nucleated cells may be examined.

Genomic DNA used for the diagnosis may be obtained from body cells, such as those present in the blood, tissue biopsy, surgical specimen, or autopsy material. The DNA may be isolated and used directly for detection of a specific sequence or may be amplified by the polymerase chain reaction (PCR) prior to analysis.

- 5 Similarly, RNA or cDNA may also be used, with or without PCR amplification. To detect a specific nucleic acid sequence, direct nucleotide sequencing, hybridization using specific oligonucleotides, restriction enzyme digest and mapping, PCR mapping, RNase protection, chemical mismatch cleavage, ligase-mediated detection, and various other methods may be employed. Oligonucleotides specific to particular
10 sequences can be chemically synthesized and labeled radioactively or non-radioactively (e.g., biotin tags, ethidium bromide), and hybridized to individual samples immobilized on membranes or other solid-supports (e.g., by dot-blot or transfer from gels after electrophoresis), or in solution. The presence or absence of the target sequences may then be visualized using methods such as autoradiography,
15 fluorometry, or colorimetry. These procedures can be automated using redundant, short oligonucleotides of known sequence fixed in high density to silicon chips.

(1) Appropriate Probes and Primers

- Whether for hybridization, RNase protection, ligase-mediated detection, PCR amplification or any other standards methods described herein and well known in the
20 art, a variety of subsequences of the PS-interacting protein sequences disclosed or otherwise enabled herein will be useful as probes and/or primers. These sequences or subsequences will include both normal sequences and deleterious mutant sequences. In general, useful sequences will include at least 8-9, more preferably 10-50, and most preferably 18-24 consecutive nucleotides from introns, exons or intron/exon
25 boundaries. Depending upon the target sequence, the specificity required, and future technological developments, shorter sequences may also have utility. Therefore, any PS-interacting protein derived sequence which is employed to isolate, clone, amplify, identify or otherwise manipulate a PS-interacting protein sequence may be regarded as an appropriate probe or primer. Particularly contemplated as useful will be sequences

including nucleotide positions from the PS-interacting protein genes in which disease-causing mutations are known to be present, or sequences which flank these positions.

(2) Hybridization Screening

For in situ detection of a normal or mutant PS-interacting protein-related
5 nucleic acid sequence, a sample of tissue may be prepared by standard techniques and then contacted with one or more of the above-described probes, preferably one which is labeled to facilitate detection, and an assay for nucleic acid hybridization is conducted under stringent conditions which permit hybridization only between the probe and highly or perfectly complementary sequences. Because many mutations
10 consist of a single nucleotide substitution, high stringency hybridization conditions may be required to distinguish normal sequences from most mutant sequences. When the PS-interacting protein genotypes of the subject's parents are known, probes may be chosen accordingly. Alternatively, probes to a variety of mutants may be employed sequentially or in combination. Because most individuals carrying
15 mutations in the PS-interacting proteins will be heterozygous, probes to normal sequences also may be employed and homozygous normal individuals may be distinguished from mutant heterozygotes by the amount of binding (e.g., by intensity of radioactive signal). In another variation, competitive binding assays may be employed in which both normal and mutant probes are used but only one is labeled.

20 (3) Restriction Mapping

Sequence alterations may also create or destroy fortuitous restriction enzyme recognition sites which are revealed by the use of appropriate enzyme digestion followed by gel-blot hybridization. DNA fragments carrying the site (normal or mutant) are detected by their increase or reduction in size, or by the increase or
25 decrease of corresponding restriction fragment numbers. Such restriction fragment length polymorphism analysis (RFLP), or restriction mapping, may be employed with genomic DNA, mRNA or cDNA. The PS-interacting protein sequences may be amplified by PCR using the above-described primers prior to restriction, in which case the lengths of the PCR products may indicate the presence or absence of
30 particular restriction sites, and/or may be subjected to restriction after amplification.

The restriction fragments may be visualized by any convenient means (e.g., under UV light in the presence of ethidium bromide).

(4) PCR Mapping

In another series of embodiments, a single base substitution mutation may be
5 detected based on differential PCR product length or production in PCR. Thus,
primers which span mutant sites or which, preferably, have 3' termini at mutation
sites, may be employed to amplify a sample of genomic DNA, mRNA or cDNA from
a subject. A mismatch at a mutational site may be expected to alter the ability of the
normal or mutant primers to promote the polymerase reaction and, thereby, result in
10 product profiles which differ between normal subjects and heterozygous and/or
homozygous mutants. The PCR products of the normal and mutant gene may be
differentially separated and detected by standard techniques, such as polyacrylamide
or agarose gel electrophoresis and visualization with labeled probes, ethidium
bromide or the like. Because of possible non-specific priming or readthrough of
15 mutation sites, as well as the fact that most carriers of mutant alleles will be
heterozygous, the power of this technique may be low.

(5) Electrophoretic Mobility

Genetic testing based on DNA sequence differences also may be achieved by
detection of alterations in electrophoretic mobility of DNA, mRNA or cDNA
20 fragments in gels. Small sequence deletions and insertions, for example, can be
visualized by high resolution gel electrophoresis of single or double stranded DNA, or
as changes in the migration pattern of DNA heteroduplexes in non-denaturing gel
electrophoresis. Mutations or polymorphisms in the PS-interacting protein genes may
also be detected by methods which exploit mobility shifts due to single-stranded
25 conformational polymorphisms (SSCP) associated with mRNA or single-stranded
DNA secondary structures.

(6) Chemical Cleavage of Mismatches

Mutations in the PS-interacting protein genes may also be detected by
employing the chemical cleavage of mismatch (CCM) method (see, e.g., Saleeba and
30 Cotton, 1993, and references therein). In this technique, probes (up to ~ 1 kb) may be

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mixed with a sample of genomic DNA, cDNA or mRNA obtained from a subject. The sample and probes are mixed and subjected to conditions which allow for heteroduplex formation (if any). Preferably, both the probe and sample nucleic acids are double-stranded, or the probe and sample may be PCR amplified together, to ensure creation of all possible mismatch heteroduplexes. Mismatched T residues are reactive to osmium tetroxide and mismatched C residues are reactive to hydroxylamine. Because each mismatched A will be accompanied by a mismatched T, and each mismatched G will be accompanied by a mismatched C, any nucleotide differences between the probe and sample (including small insertions or deletions) will lead to the formation of at least one reactive heteroduplex. After treatment with osmium tetroxide and/or hydroxylamine to modify any mismatch sites, the mixture is subjected to chemical cleavage at any modified mismatch sites by, for example, reaction with piperidine. The mixture may then be analyzed by standard techniques such as gel electrophoresis to detect cleavage products which would indicate mismatches between the probe and sample.

(7) Other Methods

Various other methods of detecting PS-interacting protein mutations, based upon the sequences disclosed and otherwise enabled herein, will be apparent to those of ordinary skill in the art. Any of these may be employed in accordance with the present invention. These include, but are not limited to, nuclease protection assays (S1 or ligase-mediated), ligated PCR, denaturing gradient gel electrophoresis (DGGE; see, e.g., Fischer and Lerman, 1983), restriction endonuclease fingerprinting combined with SSCP (REF-SSCP; see, e.g., Liu and Sommer, 1995), and the like.

D. Other Screens and Diagnostics

In inherited cases, as the primary event, and in non-inherited cases as a secondary event due to the disease state, abnormal processing of the presenilins, PS-interacting proteins, APP, or proteins reacting with the presenilins, PS-interacting proteins, or APP may occur. This can be detected as abnormal phosphorylation, glycosylation, glycation amidation or proteolytic cleavage products in body tissues or fluids (e.g., CSF or blood).

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Diagnosis also can be made by observation of alterations in transcription, translation, and post-translational modification and processing, as well as alterations in the intracellular and extracellular trafficking of gene products in the brain and peripheral cells. Such changes will include alterations in the amount of messenger
5 RNA and/or protein, alteration in phosphorylation state, abnormal intracellular location/distribution, abnormal extracellular distribution, etc. Such assays will include: Northern Blots (e.g., with PS-interacting protein-specific and non-specific nucleotide probes), Western blots and enzyme-linked immunosorbent assays (ELISA) (e.g., with antibodies raised specifically to a PS-interacting protein or PS-interacting
10 functional domain, including various post-translational modification states including glycosylated and phosphorylated isoforms). These assays can be performed on peripheral tissues (e.g., blood cells, plasma, cultured or other fibroblast tissues, etc.) as well as on biopsies of CNS tissues obtained antemortem or postmortem, and upon cerebrospinal fluid. Such assays might also include in situ hybridization and
15 immunohistochemistry (to localize messenger RNA and protein to specific subcellular compartments and/or within neuropathological structures associated with these diseases such as neurofibrillary tangles and amyloid plaques).

E. Screening and Diagnostic Kits

In accordance with the present invention, diagnostic kits are also provided
20 which will include the reagents necessary for the above-described diagnostic screens. For example, kits may be provided which include antibodies or sets of antibodies which are specific to one or more mutant epitopes. These antibodies may, in particular, be labeled by any of the standard means which facilitate visualization of binding. Alternatively, kits may be provided in which oligonucleotide probes or PCR
25 primers, as described above, are present for the detection and/or amplification of normal or mutant presenilin and/or PS-interacting protein nucleotide sequences. Again, such probes may be labeled for easier detection of specific hybridization. As appropriate to the various diagnostic embodiments described above, the oligonucleotide probes or antibodies in such kits may be immobilized to substrates
30 and appropriate controls may be provided.

11. Methods of Treatment

The present invention now provides a basis for therapeutic intervention in diseases which are caused, or which may be caused, by mutations in the PS-interacting proteins. As noted above, mutations in the hPS1 and hPS2 genes have been associated with the development of early onset forms of Alzheimer's Disease and, therefore, the present invention is particularly directed to the treatment of subjects diagnosed with, or at risk of developing, Alzheimer's Disease.

Without being bound to any particular theory of the invention, the effect of the Alzheimer's Disease related mutations in the presenilins appears to be a gain of a novel function, or an acceleration of a normal function, which directly or indirectly causes aberrant processing of the Amyloid Precursor Protein (APP) into A β peptide, abnormal phosphorylation homeostasis, and/or abnormal apoptosis in the brain. Such a gain of function or acceleration of function model would be consistent with the adult onset of the symptoms and the dominant inheritance of Alzheimer's Disease. Nonetheless, the mechanism by which mutations in the presenilins may cause these effects remains unknown.

The present invention, by identifying a set of PS-interacting proteins, provides new therapeutic targets for intervening in the etiology of presenilin-related AD. In addition, as mutations in the presenilins may cause AD, it is likely that mutations in the PS-interacting proteins may also cause AD. The fact that the PS-interacting protein S5a is alternately processed in the brains of victims of sporadic AD, as well as in the brains of victims of presenilin-linked AD, suggests that, at the very least, this PS-interacting protein is involved in the etiology of AD independent of mutations in the presenilins. It is likely that the other PS-interacting proteins also may be involved in non-presenilin-linked AD.

Therapies to treat PS-interacting protein-associated diseases such as AD may be based upon (1) administration of normal PS-interacting proteins, (2) gene therapy with normal PS-interacting protein genes to compensate for or replace the mutant genes, (3) gene therapy based upon antisense sequences to mutant PS-

interacting protein genes or which "knock-out" the mutant genes, (4) gene therapy based upon sequences which encode a protein which blocks or corrects the deleterious effects of PS-interacting protein mutants, (5) immunotherapy based upon antibodies to normal and/or mutant PS-interacting proteins, or (6) small molecules (drugs) which
5 alter PS-interacting protein expression, alter interactions between PS-interacting proteins and other proteins or ligands, or which otherwise block the aberrant function of mutant presenilin or PS-interacting proteins by altering the structure of the mutant proteins, by enhancing their metabolic clearance, or by inhibiting their function.

A. Protein Therapy

10 Treatment of Alzheimer's Disease, or other disorders resulting from PS-interacting protein mutations, may be performed by replacing the mutant protein with normal protein, by modulating the function of the mutant protein, or by providing an excess of normal protein to reduce the effect of any aberrant function of the mutant proteins.

15 To accomplish this, it is necessary to obtain, as described and enabled herein, large amounts of substantially pure PS-interacting protein from cultured cell systems which can express the protein. Delivery of the protein to the affected brain areas or other tissues can then be accomplished using appropriate packaging or administration systems including, for example, liposome mediated protein delivery to
20 the target cells.

B. Gene Therapy

In one series of embodiments, gene therapy may be employed in which normal copies of a PS-interacting protein gene are introduced into patients to code successfully for normal protein in one or more different affected cell types. The gene
25 must be delivered to those cells in a form in which it can be taken up and code for sufficient protein to provide effective function. Thus, it is preferred that the recombinant gene be operably joined to a strong promoter so as to provide a high level of expression which will compensate for, or out-compete, the mutant proteins. As noted above, the recombinant construct may contain endogenous or exogenous

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regulatory elements, inducible or repressible regulatory elements, or tissue-specific regulatory elements.

In another series of embodiments, gene therapy may be employed to replace the mutant gene by homologous recombination with a recombinant construct.

- 5 The recombinant construct may contain a normal copy of the targeted PS-interacting protein gene, in which case the defect is corrected in situ, or may contain a "knock-out" construct which introduces a stop codon, missense mutation, or deletion which abolished function of the mutant gene. It should be noted in this respect that such a construct may knock-out both the normal and mutant copies of the targeted gene in a
- 10 heterozygous individual, but the total loss of gene function may be less deleterious to the individual than continued progression of the disease state.

- In another series of embodiments, antisense gene therapy may be employed. The antisense therapy is based on the fact that sequence-specific suppression of gene expression can be achieved by intracellular hybridization between
- 15 mRNA or DNA and a complementary antisense species. The formation of a hybrid duplex may then interfere with the transcription of the gene and/or the processing, transport, translation and/or stability of the target mRNA. Antisense strategies may use a variety of approaches including the administration of antisense oligonucleotides or antisense oligonucleotide analogs (e.g., analogs with phosphorothioate backbones)
- 20 or transfection with antisense RNA expression vectors. Again, such vectors may include exogenous or endogenous regulatory regions, inducible or repressible regulatory elements, or tissue-specific regulatory elements.

- In another series of embodiments, gene therapy may be used to introduce a recombinant construct encoding a protein or peptide which blocks or otherwise
- 25 corrects the aberrant function caused by a mutant presenilin or PS-interacting protein gene. In one embodiment, the recombinant gene may encode a peptide which corresponds to a domain of a PS-interacting which has been found to abnormally interact with another cell protein or other cell ligand (e.g., a mutant presenilin). Thus, for example, if a mutant PS1 TM6→7 domain is found to interact with a PS-
- 30 interacting protein but the corresponding normal TM6→7 domain does not undergo

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this interaction, gene therapy may be employed to provide an excess of the mutant TM6→7 domain which may compete with the mutant presenilin protein and inhibit or block the aberrant interaction. Alternatively, the PS-interacting domain of a PS-interacting protein which interacts with a mutant, but not a normal, presenilin may be encoded and expressed by a recombinant construct in order to compete with, and thereby inhibit or block, the aberrant interaction.

Retroviral vectors can be used for somatic cell gene therapy especially because of their high efficiency of infection and stable integration and expression. A full length PS-interacting protein gene, subsequences encoding functional domains of these proteins, or any of the other therapeutic peptides described above, can be cloned into a retroviral vector and expression may be driven from its endogenous promoter, from the retroviral long terminal repeat, or from a promoter specific for the target cell type of interest (e.g., neurons). Other viral vectors which can be used include adeno-associated virus, vaccinia virus, bovine papilloma virus, or a herpes virus such as Epstein-Barr virus.

C. Immunotherapy

Immunotherapy is also possible for Alzheimer's Disease. Antibodies may be raised to a normal or mutant PS-interacting protein (or a portion thereof) and are administered to the patient to bind or block an aberrant interaction (e.g., with a mutant presenilin) and prevent its deleterious effects. Simultaneously, expression of the normal protein product could be encouraged. Alternatively, antibodies may be raised to specific complexes between mutant or wild-type PS-interacting proteins and their interaction partners.

A further approach is to stimulate endogenous antibody production to the desired antigen. Administration could be in the form of a one time immunogenic preparation or vaccine immunization. The PS-interacting protein or other antigen may be mixed with pharmaceutically acceptable carriers or excipients compatible with the protein. The immunogenic composition and vaccine may further contain auxiliary substances such as emulsifying agents or adjuvants to enhance effectiveness.

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Immunogenic compositions and vaccines may be administered parenterally by injection subcutaneously or intramuscularly.

D. Small Molecule Therapeutics

As described and enabled herein, the present invention provides for a number of methods of identifying small molecules or other compounds which may be useful in the treatment of Alzheimer's Disease or other disorders caused by mutations in the presenilins or PS-interacting proteins. Thus, for example, the present invention provides for methods of identifying proteins which bind to normal or mutant PS-interacting proteins (aside from the presenilins). The invention also provides for methods of identifying small molecules which can be used to disrupt aberrant interactions between mutant presenilins and/or PS-interacting proteins and such other binding proteins or other cell components.

Examples

15 Example 1. Isolation of PS-interacting proteins by two-hybrid yeast system.

To identify proteins interacting with the presenilin proteins, a commercially available yeast two-hybrid kit ("Matchmaker System 2" from Clontech, Palo Alto, CA) was employed to screen a brain cDNA library for clones which interact with functional domains of the presenilins. In view of the likelihood that the TM6→7 loop domains of the presenilins are important functional domains, partial cDNA sequences encoding either residues 266-409 of the normal PS1 protein or residues 272-390 of the normal PS2 protein were ligated in-frame into the EcoRI and BamHI sites of the pAS2-1 fusion-protein expression vector (Clontech). The resultant fusion proteins contain the GAL4 DNA binding domain coupled in-frame either to the TM6→7 loop of the PS1 protein or to the TM6→7 loop of the PS2 protein. These expression plasmids were co-transformed into *S. cerevisiae* strain Y190 together with a library of human brain cDNAs ligated into the pACT2 yeast fusion-protein expression vector (Clontech) bearing the GAL4 activation domain using modified lithium acetate protocols of the "Matchmaker System 2" yeast two-hybrid kit (Clontech, Palo Alto, CA). Yeast clones bearing human brain cDNAs which interact

with the TM6→7 loop domain were selected for His⁻ resistance by plating on SD minimal medium lacking histidine and for βgal⁺ activation by color selection. The His⁺ βgal⁺ clones were then purged of the pAS2-1 "bait" construct by culture in 10 μg/ml cyclohexamide and the unknown "trapped" inserts of the human brain cDNAs encoding PS-interacting proteins were isolated by PCR and sequenced. Of 6 million initial transformants, 200 positive clones were obtained after His⁻ selection, and 42 after βgal⁺ color selection, carried out in accordance with the manufacturer's protocol for selection of positive colonies. Of these 42 clones there were several independent clones representing the same genes.

To address the likelihood that mutations in the presenilins cause AD through the acquisition of a novel but toxic function (i.e., dominant gain of function mutation) which is mediated by a novel interaction between the mutant proteins and one or more other cellular proteins, the human brain cDNA library cloned into the pACT2 expression vector (Clontech) was re-screened using mutant TM6→7 loop domain sequences as described above and according to manufacturer's protocols. In particular, mutant presenilin sequences corresponding to residues 260-409 of PS1 TM6→7 loop domains bearing mutations L286V, L392V and Δ290-319 were ligated in-frame into the GAL4 DNA-binding domain of the pAS2-1 vector (Clontech) and used to screen the human brain cDNA:GAL4 activation domain library of pACT vectors (Clontech). Yeast were co-transformed, positive colonies were selected, and "trapped" sequences were recovered and sequenced as described above. In addition to some of the same sequences recovered with the normal TM6→7 loop domains, several new sequences were obtained which reflect aberrant interactions of the mutant presenilins with normal cellular proteins.

The recovered and sequenced clones corresponding to these PS-interacting proteins were compared to the public sequence databases using the BL:ASTN algorithm via the NCBI e-mail server. Descriptions of several of these clones follow:

Antisecretory Factor/ Proteasome S5a Subunit. Two overlapping clones (Y2H29 and Y2H31) were identified which correspond to a C-terminal fragment of a protein alternatively identified as Antisecretory Factor ("ASF") or the Multiubiquitin

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chain binding S5a subunit of the 26S proteasome ("S5a") (Johansson et al. 1995; Ferrell et al., 1996). The complete nucleotide and amino acid sequences of the S5a subunit are available through the public databases under Accession number U51007 and are reproduced here as SEQ ID NO: 1 and SEQ ID NO: 2. The nucleotide
5 sequences of the Y2H29 and Y2H31 clones include nucleotides 351-1330 of SEQ ID NO: 1 and amino acid residues 70-377 of SEQ ID NO: 2. Thus, residues 70-377 of the full S5a subunit include the PS-interacting domain of this protein. Residues 206-377 of S5a contain certain motifs that are important for protein-protein interactions (Ferrell et al., 1996).

10 The PS1-S5a subunit interaction was directly re-tested for both wild type and mutant PS1 TM6→7 loop (residues 260-409) by transforming Y187 yeast cells with the appropriate wild type or mutant (L286V, L392V or Δ 290-319) cDNA ligated in-frame to the GAL4-DNA binding domain of pACT2. The Δ 290-319 mutant fusion construct displayed autonomous β gal activation in the absence of any S5a "target
15 sequence" and, therefore, could not be further analyzed. In contrast, both the L286V and L392V mutant constructs interacted specifically with the S5a construct. Quantitative assays, however, showed that these interactions were weaker than those involving the wild type PS1₂₆₀₋₄₀₉ sequence and that the degree of interaction was crudely correlated with the age of onset of FAD. The difference in β gal activation
20 was not attributable to instability of the mutant PS1₂₆₀₋₄₀₉ construct mRNAs or fusion proteins because Western blots of lysates of transformed yeast showed equivalent quantities of mutant or wild-type fusion proteins.

Because one of the putative functions of S5a is to bind multi-ubiquitinated proteins, the PS1:S5a interaction observed in S. cerevisiae could arise either through
25 yeast-dependent ubiquitination of the PS1₂₆₀₋₄₀₉ construct, or by direct interaction. The former would reflect a degradative pathway, a functional and perhaps reciprocal interaction between PS1 and S5a, or both. A direct interaction is favored by the fact that the PS1:S5a interaction is decreased rather than increased by the presence of the L286V and L392V mutations, and by the fact that neither of these mutations affect
30 ubiquitin conjugation sites in the PS1₂₆₀₋₄₀₉ loop (i.e., K265, K311, K314 or K395).

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To further examine this possibility, we investigated the direct interaction of recombinant His-tagged fusion proteins corresponding to full length S5a and the PS1₂₆₀₋₄₀₉ loop. Partially purified recombinant His-tagged PS1₂₆₀₋₄₀₉ loop and His-tagged S5a proteins and appropriate controls were mixed in phosphate buffered saline.

5 The mixture was then subjected to size exclusion chromatography, and eluates were examined by SDS-PAGE and Western blotting using anti-His-tag monoclonal antibodies (Quiagen). In the crude PS1₂₆₀₋₄₀₉ loop preparation alone, the PS1₂₆₀₋₄₀₉ loop eluted from the size exclusion column as a broad peak at 35 minutes. In the crude S5a preparation alone, S5a eluted at 25 minutes. However, when the crude PS1₂₆₀₋₄₀₉ loop and S5a preparations were mixed, there was a significant shift in the elution of PS1₂₆₀₋₄₀₉ toward a higher molecular weight complex. Co-elution of S5a and PS1₂₆₀₋₄₀₉ in the same fraction was confirmed by SDS-PAGE and Western blotting of fractions using the anti-His-tag antibody. These results are consistent with a ubiquitin-independent and, therefore, possibly functional interaction.

15 GT24 and related genes with homology to p120/plakoglobin family. Five over-lapping clones (Y2H6, Y2H10b, Y2H17h2, Y2H24, and Y2H25) were obtained which interact with the normal PS1 TM6→7 loop domain and which appear to represent at least one novel gene. The Y2H24 clone was also found to interact with the mutant PS1 TM6→7 loop domains. Note that it appears that more than one member of the gene family was isolated, suggesting a family of genes interacting differentially with different presenilins. The most complete available cDNA corresponding to these clones was designated GT24 and is disclosed herein as SEQ ID NO: 3 and has been deposited with GenBank as Accession number U81004. The open reading frame suggests that GT24 is a protein of at least 1040 amino acids with a unique N-terminus, and considerable homology to several armadillo (arm) repeat proteins at its C-terminus. The predicted amino acid sequence of GT24 is disclosed herein as SEQ ID NO: 4. Thus, for example, residues 440-862 of GT24 have 32-56% identity ($p=1.2e^{-133}$) to residues 440-854 of murine p120 protein (Accession number Z17804), and residues 367-815 of GT24 have 26-42% identity ($p=0.0017$) to residues 245-465 of the D. melanogaster armadillo segment polarity protein (Accession

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number P18824). The GT24 gene maps to chromosome 5p15 near the anonymous microsatellite marker D5S748 and the Cri-du-Chat syndrome locus. This sequence is also nearly identical to portions of two human ESTs of unknown function (i.e., nucleotides 2701-3018 of Accession number F08730 and nucleotides 2974-3348 of Accession number T18858). These clones also show lower degrees of homology with other partial cDNA and gDNA sequences (e.g., H17245, T06654, T77214, H24294, M62015, T87427 and G04019).

p0071 gene. An additional His⁻, β gal⁺ clone isolated in the initial screening with wild type PS1₂₆₆₋₄₀₉ "bait" had a similar nucleotide sequence to GT24 (target clone Y2H25; Accession number U81005), and would also be predicted to encode a peptide with C-terminal arm repeats. A longer cDNA sequence closely corresponding to the Y2H25 clone has been deposited in GenBank as human protein p0071 (Accession number X81889). The nucleotide and corresponding amino acid sequences of p0071 are reproduced herein as SEQ ID NOs: 5 and 6. Comparison of the predicted sequence of the p0071 ORF with that of GT24 confirms that they are related proteins with 47% overall amino acid sequence identity, and with 70% identity between residues 346-862 of GT24, and residues 509-1022 of p0071 (which includes residues encoded by the Y2H25 cDNA). The latter result strongly suggests that PS1 interacts with a novel class of arm repeat containing proteins. The broad ~ 4 kb hybridization signal obtained on Northern blots with the unique 5' end of GT24 could reflect either alternate splicing/polyadenylation of GT24, or the existence of additional members of this family with higher degrees of N-terminal homology to GT24 than p0071.

Rab11 gene. This clone (Y2H9), disclosed herein as SEQ ID NO: 7, was identified as interacting with the normal PS1 TM6→7 loop domain and appears to correspond to a known gene, Rab11, available through Accession numbers X56740 and X53143. Rab11 is believed to be involved in protein/vesicle trafficking in the ER/Golgi. Note the possible relationship to processing of membrane proteins such as β APP and Notch with resultant overproduction of toxic A β peptides (especially neurotoxic A β ₁₋₄₂₍₄₃₎ isoforms) (Scheuner, et al, 1995).

Retinoid X receptor- β gene. This clone (Y2H23b), disclosed herein as SEQ ID NO: 8, was identified as interacting with the normal PS1 TM6 \rightarrow 7 loop domain and appears to correspond to a known gene, known variously as the retinoid X receptor- β , nuclear receptor co-regulator or MHC Class I regulatory element, and
5 available through Accession numbers M84820, X63522 and M81766. This gene is believed to be involved in intercellular signaling, suggesting a possible relationship to the intercellular signaling function mediated by C. elegans sel12 and Notch/lin-12 (transcription activator).

Cytoplasmic chaperonin gene. This clone (Y2H27), disclosed herein as
10 SEQ ID NO: 9, was identified as interacting with the normal PS1 TM6 \rightarrow 7 loop domain and appears to correspond to a known gene, a cytoplasmic chaperonin containing TCP-1, available through Accession numbers U17104 and X74801.

Unknown gene (Y2H35). This clone (Y2H35), disclosed herein as SEQ ID NO: 10, was identified as interacting with the normal PS1 TM6 \rightarrow 7 loop domain
15 and appears to correspond to a known gene of unknown function, available through Accession number R12984, which shows conservation down through yeast.

Unknown gene (Y2H171). This clone (Y2H171), disclosed herein as SEQ ID NO: 11, was identified as interacting with the normal PS1 TM6 \rightarrow 7 loop domain and appears to correspond to a known expressed repeat sequence available through
20 Accession number D55326.

Unknown gene (Y2H41). This clone (Y2H41) was identified which reacts strongly with the TM6 \rightarrow 7 loop domains of both PS1 and PS2 as well as the mutant loop domains of PS1. The sequence, disclosed as SEQ ID NO: 12, shows strong homology to an EST of unknown function (Accession number T64843).

25 Example 2. Isolation of presenilin binding proteins by affinity chromatography.

To identify the proteins which may be involved in the biochemical function of the presenilins, PS-interacting proteins were isolated using affinity chromatography. A GST-fusion protein containing the PS1 TM6 \rightarrow 7 loop, prepared as described in Example 3, was used to probe human brain extracts, prepared by
30 homogenizing brain tissue by Polytron in physiological salt solution. Non-specific

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binding was eliminated by pre-clearing the brain homogenates of endogenous GST-binding components by incubation with glutathione-Sepharose beads. These GST-free homogenates were then incubated with the GST-PS fusion proteins to produce the desired complexes with functional binding proteins. These complexes were then
5 recovered using the affinity glutathione-Sepharose beads. After extensive washing with phosphate buffered saline, the isolated collection of proteins was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Tris-tricine gradient gel 4-20%). Two major bands were observed at ~14 and 20 kD in addition to several weaker bands ranging from 50 to 60 kD.

10 The same approach may now be used to identify proteins which have binding activity for the PS-interacting proteins and, thereby, to further elucidate the etiology of AD and to identify additional therapeutics targets for intervention in AD and related disorders.

Example 3. Eukaryotic and prokaryotic expression vector systems.

15 Constructs suitable for use in eukaryotic and prokaryotic expression systems have been generated using different classes of PS1 nucleotide cDNA sequence inserts. In the first class, termed full-length constructs, the entire PS1 cDNA sequence is inserted into the expression plasmid in the correct orientation, and includes both the natural 5' UTR and 3' UTR sequences as well as the entire open
20 reading frame. The open reading frames bear a nucleotide sequence cassette which allows either the wild type open reading frame to be included in the expression system or alternatively, single or a combination of double mutations can be inserted into the open reading frame. This was accomplished by removing a restriction fragment from the wild type open reading frame using the enzymes NarI and PflmI and replacing it
25 with a similar fragment generated by reverse transcriptase PCR and bearing the nucleotide sequence encoding either the M146L mutation or the H163R mutation. A second restriction fragment was removed from the wild type normal nucleotide sequence for the open reading frame by cleavage with the enzymes PflmI and NcoI and replaced with a restriction fragment bearing the nucleotide sequence encoding the
30 A246E mutation, the A260V mutation, the A285V mutation, the L286V mutation, the

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L392V mutation or the C410Y mutation. A third variant, bearing a combination of either the M146L or H163R mutation in tandem with one of the remaining mutations, was made by linking a NarI-PflmI fragment bearing one of the former mutations and a PflmI-NcoI fragment bearing one of the latter mutations.

5 The second class of cDNA inserts, termed truncated constructs, was constructed by removing the 5' UTR and part of the 3' UTR sequences from full length wild type or mutant cDNA sequences. The 5' UTR sequence was replaced with a synthetic oligonucleotide containing a KpnI restriction site (GGTAC/C) and a small sequence (GCCACC) to create a Kozak initiation site around the ATG at the
10 beginning of the PS1 ORF. The 3' UTR was replaced with an oligonucleotide with an artificial EcoRI site at the 5' end. Mutant variants of this construct were then made by inserting the mutant sequences described above at the NarI-PflmI and PflmI-NcoI sites as described above.

 For eukaryotic expression, these various cDNA constructs bearing wild
15 type and mutant sequences, as described above, were cloned into the expression vector pZeoSV in which the SV60 promoter cassette had been removed by restriction digestion and replaced with the CMV promoter element of pcDNA3 (Invitrogen). For prokaryotic expression, constructs have been made using the glutathione S-transferase (GST) fusion vector pGEX-kg. The inserts which have been attached to the GST
20 fusion nucleotide sequence are the same nucleotide sequences described above bearing either the normal open reading frame nucleotide sequence, or bearing a combination of single and double mutations as described above. These GST fusion constructs allow expression of the partial or full-length protein in prokaryotic cell systems as mutant or wild type GST fusion proteins, thus allowing purification of the
25 full-length protein followed by removal of the GST fusion product by thrombin digestion. A further cDNA construct was made with the GST fusion vector, to allow the production of the amino acid sequence corresponding to the hydrophilic acidic loop domain between TM6 and TM7 of the full-length protein, either as a wild type nucleotide sequence or as a mutant sequence bearing either the A285V mutation, the
30 L286V mutation or the L392V mutation. This was accomplished by recovering wild

type or mutant sequence from appropriate sources of RNA using a 5' oligonucleotide primer with a 5' BamHI restriction site (G/GATCC), and a 3' primer with a 5' EcoRI restriction site (G/AATTC). This allowed cloning of the appropriate mutant or wild type nucleotide sequence corresponding to the hydrophilic acidic loop domain at the BamHI and the EcoRI sites within the pGEX-KG vector.

The PS-interacting protein genes may be similarly manipulated by recombinant means for expression in prokaryotic or eukaryotic hosts. In particular, GST or other fusion proteins may be produced which will be useful in assays (e.g., yeast two-hybrid studies) for therapeutics.

Example 4. Antibody production.

Peptide antigens corresponding to portions of the PS1 protein were synthesized by solid-phase techniques and purified by reverse phase high pressure liquid chromatography. Peptides were covalently linked to keyhole limpet hemocyanin (KLH) via disulfide linkages that were made possible by the addition of a cysteine residue at the peptide C-terminus of the presenilin fragment. This additional residue does not appear normally in the protein sequence and was included only to facilitate linkage to the KLH molecule.

A total of three New Zealand white rabbits were immunized with peptide-KLH complexes for each peptide antigen in combination with Freund's adjuvant and were subsequently given booster injections at seven day intervals. Antisera were collected for each peptide and pooled and IgG precipitated with ammonium sulfate. Antibodies were then affinity purified with Sulfo-link agarose (Pierce) coupled with the appropriate peptide. This final purification is required to remove non-specific interactions of other antibodies present in either the pre- or post-immune serum.

The specificity of each antibody was confirmed by three tests. First, each detected single predominant bands of the approximate size predicted for presenilin-1 on Western blots of brain homogenate. Second, each cross-reacted with recombinant fusion proteins bearing the appropriate sequence. Third each could be specifically blocked by pre-absorption with recombinant PS1 or the immunizing peptide.

Antibodies to peptides derived from the PS-interacting proteins may be produced by similar means.

Example 5. Transgenic mice.

A series of wild type and mutant PS1 and PS2 genes were constructed for
5 use in the preparation of transgenic mice. Mutant versions of PS1 and PS2 were generated by site-directed mutagenesis of the cloned cDNAs using standard techniques.

The cDNAs and their mutant versions were used to prepare two classes of mutant and wild type PS1 and PS2 cDNAs, as described in Example 3. The first
10 class, referred to as "full-length" cDNAs, were prepared by removing approximately 200 bp of the 3' untranslated region immediately before the polyA site by digestion with EcoRI (PS1) or PvuII (PS2). The second class, referred to as "truncated" cDNAs, were prepared by replacing the 5' untranslated region with a ribosome binding site (Kozak consensus sequence) placed immediately 5' of the ATG start
15 codon.

Various full length and truncated wild type and mutant PS1 and PS2 cDNAs, prepared as described above, were introduced into one or more of the following vectors and the resulting constructs were used as a source of gene for the production of transgenic mice.

20 The cos.TET expression vector: This vector was derived from a cosmid clone containing the Syrian hamster PrP gene. It has been described in detail by Scott et al. (1992) and Hsiao et al. (1995). PS1 and PS2 cDNAs (full length or truncated) were inserted into this vector at its SalI site. The final constructs contain 20 kb of 5' sequence flanking the inserted cDNA. This 5' flanking sequence includes the PrP
25 gene promoter, 50 bp of a PrP gene 5' untranslated region exon, a splice donor site, a 1 kb intron, and a splice acceptor site located immediately adjacent to the SalI site into which the PS1 or PS2 cDNA was inserted. The 3' sequence flanking the inserted cDNA includes an approximately 8 kb segment of PrP 3' untranslated region including a polyadenylation signal. Digestion of this construct with NotI (PS1) or
30 FseI (PS2) released a fragment containing a mutant or wild type PS gene under the

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control of the PrP promoter. The released fragment was gel purified and injected into the pronuclei of fertilized mouse eggs using the method of Hsiao et al. (1995).

Platelet-derived growth factor receptor β -subunit constructs: PS cDNAs were also introduced between the Sall (full length PS1 cDNAs) or HindIII (truncated PS1 cDNAs, full length PS2 cDNAs, and truncated PS2 cDNAs) at the 3' end of the human platelet derived growth factor receptor β -subunit promoter and the EcoRI site at the 5' end of the SV40 polyA sequence and the entire cassette was cloned into the pZcoSV vector (Invitrogen, San Diego, CA.). Fragments released by ScaI/BamHI digestion were gel purified and injected into the pronuclei of fertilized mouse eggs using the method of Hsiao et al. (1995).

Human β -actin constructs: PS1 and PS2 cDNAs were inserted into the Sall site of pBACGH. The construct produced by this insertion includes 3.4 kb of the human β actin 5' flanking sequence (the human β actin promoter, a spliced 78 bp human β actin 5' untranslated exon and intron) and the PS1 or PS2 insert followed by 2.2 kb of human growth hormone genomic sequence containing several introns and exons as well as a polyadenylation signal. SfiI was used to release a PS-containing fragment which was gel purified and injected into the pronuclei of fertilized mouse eggs using the method of Hsiao et al. (1995).

Phosphoglycerate kinase constructs: PS1 and PS2 cDNAs were introduced into the pkJ90 vector. The cDNAs were inserted between the KpnI site downstream of the human phosphoglycerate kinase promoter and the XbaI site upstream of the 3' untranslated region of the human phosphoglycerate kinase gene. PvuII/HindIII (PS1 cDNAs) or PvuII (PS2 cDNAs) digestion was used to release a PS-containing fragment which was then gel purified and injected into the pronuclei of fertilized mouse eggs as described above.

Analysis of A β in transgenic murine hippocampus: To analyze the effect of a mutant human PS1 transgene in mice, a PS1 mutation observed in conjunction with a particularly severe form of early-onset PS1-linked Alzheimer's disease was used, namely the M146L missense mutation (Sherrington et al., 1995). The animals, which were heterozygous for the PS1 mutant transgene on a mixed FVB-C57BL/6

strain background, were cross-bred with similar mice bearing the human wild-type β APP₆₉₅ cDNA under the same Syrian hamster PrP promoter similar to those animals recently described by Hsiao et al., 1995. These cross breedings were done because it is thought that human A β is more susceptible to the formation of aggregates than are murine A β peptides.

The progeny of these PS1_{M146L} x β APP_{WT} cross-breedings were then genotyped to identify animals that contained both the human wild-type β APP₆₉₅ transgene and also the mutant human PS1_{M146L} transgene. These mice were aged until two to three months of age and then sacrificed, with the hippocampus and neocortex being dissected rapidly from the brain and frozen. Litter mates of these mice, which contained only the wild-type human β APP₆₉₅ transgene were also sacrificed, and their hippocampi and neocortices were dissected and rapidly frozen as well.

The concentration of both total A β peptides (A β _{X-40} and A β _{X-42(43)}) as well as the subset of A β peptides ending on residues 42 or 43 (long-tailed A β ₄₂ peptides) were then measured using a two-sandwich ELISA as described previously (Tamaoka et al., 1994; Suzuki et al., 1994). These results convincingly showed a small increase in total A β peptides in the double transgenic animals bearing wild-type human β APP₆₉₅ and mutant human PS1_{M146L} transgenes compared to the wild-type human β APP₆₉₅ controls. More impressively, these measurements also showed that there was an increase in the amount of long-tailed A β peptides ending on residues 42 or 43 (A β ₄₂). In contrast, litter mates bearing only the wild-type human β APP₆₉₅ transgene had A β ₄₂ long-tailed peptide values which were below the limit of quantitation ("BLQ").

These observations therefore confirm that the construction of transgenic animals can recapitulate some of the biochemical features of human Alzheimer's disease (namely the overproduction of A β peptide and, in particular, overproduction of long-tailed isoforms of A β peptide). These observations thus prove that the transgenic models are in fact useful in exploring therapeutic targets relevant to the treatment and prevention of Alzheimer's disease.

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Analysis of hippocampus dependent memory functions in PS1 transgenic mice: Fourteen transgenic C57BL/6 x FVB mice bearing the human PS1_{M146V} mutant transgene under the PrP promoter (as described) above and 12 wild type litter mates aged 2.5-3 months of age (both groups were balanced for age, weight, and sex) were investigated for behavioral differences attributable to the mutant transgene. Also the qualitative observation of murine behavior in their home cages did not indicate bimodal distribution of behaviors in the sample of animals.

Experiment 1. To test for subtle differences in exploratory behavior (e.g. locomotion, scanning of the environment through rearing, and patterns of investigation of unfamiliar environment), both PS1_{M146V} and wild type litter mates were tested in the open-field (Janus, et al. 1995). The results of the test revealed no significant differences between transgenics and controls in exploration of a new environment measured by mice locomotor behaviors (walking, pausing, wall leaning, rearing, grooming), ($F(1,24) = .98$, NS). Thus, differences any in behavior on the Morris water maze test (see below) cannot be attributed to differences in locomotor abilities, etc.

Experiment 2. One week after the open-field test, the PS1_{M146V} mutant transgenic mice and their litter mates were trained in the Morris water maze. In this test, a mouse has to swim in a pool in order to find a submerged escape platform. The animal solves that test through learning the location of the platform using the available extra-maze spatial cues (Morris, 1990). This test was chosen because there is strong evidence that the hippocampal formation is involved in this form of learning. The hippocampus is also a major site of AD neuropathology in humans and defects in spatial learning (geographic disorientation, losing objects, wandering, etc.) are prominent early features of human AD. As a result the test is likely to detect early changes equivalent to those seen in human AD. The Morris test is conducted in three phases. In the first phase (the learning acquisition phase), the mouse has to learn the spatial position of the platform. In the second phase (the probe trial), the platform is removed from the pool and the mouse's search for the platform is recorded. In the final phase (the learning transfer phase), the platform is replaced in

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a new position in the pool, and the mouse has to learn that new spatial position of the platform.

Transgenic and wild type mice did not differ in their latencies to find the platform during learning acquisition ($F(1,24) = 0.81$, NS), and both groups showed rapid learning across trials ($F(10,15) = 11.57$, $p < 0.001$). During the probe trial phase, mice from both groups searched the quadrant of the pool which originally contained the platform significantly longer than other areas of the pool which had not contained the platform ($F(3,22) = 28.9$, $p < 0.001$). However, the wild type controls showed a trend which was not quite statistically significant ($t(24) = 1.21$, $p = 0.24$) for an increased number of crossings of the exact previous position of the platform. In the learning transfer test, both groups showed the same latency of finding the new position of the platform in the initial block of trials ($t(24) = 1.11$, NS). Such long latency to find the new spatial position is expected because the mice spent most of their time searching for the platform in the old spatial position. However, in later trials in the learning transfer phase, the wild type mice showed shorter swim latencies to the new position of the platform compared to the $PS1_{M146V}$ mutant transgenics ($F(1,24) = 2.36$, $p = 0.14$). The results indicate that $PS1_{M146V}$ mutant transgenic mice were less flexible in transferring learned information to a new situation and tended to persevere in their search for the platform in the old location.

Thus, although no differences were found in the spontaneous exploration of a new environment and in the acquisition of new spatial information between the wild type and the $PS1_{M146V}$ mutant transgenic mice, the $PS1_{M146V}$ mutant transgenic mice were impaired in switching and/or adapting this knowledge in later situations.

Electrophysiological Recordings in the hippocampus of mutant transgenic mice: Five to six months old litter mate control and human $PS1_{M146V}$ mutant transgenic mice on the same C57BL/6 x FVB strain backgrounds as above were used to study long term potentiation (LTP) as an electrophysiologic correlate of learning and memory in the hippocampus. Recordings were carried out on 400 μ m thick hippocampal slices according to conventional techniques. Briefly, brains were removed and transverse sections containing hippocampi were obtained within 1 min.

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after mice were decapitated under halothane anesthesia. Slices were kept at room temperature in oxygenated artificial cerebrospinal fluid for one hour prior to recording. One slice at a time was transferred to the recording chamber, where they were maintained at 32 °C in an interface between oxygenated artificial cerebrospinal
5 fluid and humidified air. Slices were then allowed to equilibrate in the recording chamber for another hour.

Extracellular field recordings were carried out in the CA1 subfield of the hippocampus at the Schaeffer collateral-pyramidal cell synapse. Synaptic responses were induced by the stimulation of Schaeffer collaterals at a frequency of 0.03 Hz and
10 an intensity of 30-50 % of maximal response. Tetani to evoke long-term potentiation consisted of 5 trains of 100 Hz stimulation lasting for 200 ms at an intertrain interval of 10 seconds. Field potentials were recorded using an Axopatch 200B amplifier (Axon Instrument). Glass pipettes were fabricated from borosilicate glass with an outer diameter of 1.5 mm, and pulled with a two step Narishige puller. Data were
15 acquired on a 486-IBM compatible computer using PCLAMP6 software (Axon Instrument).

To test for any abnormality in presynaptic function, we investigated the differences in paired-pulse facilitation, which is an example of use-dependent increase in synaptic efficacy and is considered to be presynaptic in origin. In hippocampus,
20 when two stimuli are delivered to the Schaeffer collaterals in rapid succession, paired-pulse facilitation manifests itself as an enhanced dendritic response to the second stimulus as the interstimulus interval gets shorter. In three pairs of wild-type/transgenic mice, we did not observe any difference in the paired-pulse facilitation over an interstimulus interval range of 20 ms to 1 sec. These data suggest that in
25 PS1_{M146V} mutant transgenic mice, the excitability of Schaeffer collateral fibers and neurotransmitter release are likely to be normal.

Tetanic stimulation induced a long-lasting increase in the synaptic strength in both control (n = 3) and PS1_{M146V} mutant transgenic mice (n = 2). In slices obtained from the PS1_{M146V} mutant transgenic mice, long-lasting increase in the synaptic
30 strength was 30 % more than that obtained from control mice.

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Although preferred embodiments of the invention have been described herein in detail, it will be understood by those skilled in the art that variations may be made thereto without departing from the spirit of the invention or the scope of the
5 appended claims.

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SEQUENCE LISTING

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(B) FILING DATE: 02-JAN-1997

(xii) ATTORNEY/AGENT INFORMATION:

(A) NAME: RAE, Patricia A.

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (416) 595-1155
(B) TELEFAX: (416) 595-1163

(2) INFORMATION FOR SEQ ID NO:1:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1330 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 145..1275
(D) OTHER INFORMATION: /product= "S5a"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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AATTCCTTCTTA TGACCTTTTAT TTTCATACAG AGATACAAAG GCAACTATGT GCAGCAACAA      60
TCTGATGGGC AGTCCAAACT CTGGGAGGA AATAAATTCA TGGTAAATGT CATGATGGCG      120
GTGGGAGGG AGGAAGCTGG CAAG ATG GTG TTG GAA AGC ACT ATG GTG TGT      171
Met Val Leu Glu Ser Thr Met Val Cys
1      5
GTG GAC AAC AGT GAG TAT ATG CGG AAT GGA GAC TTC TTA CCC ACC AGG      219
Val Asp Asn Ser Glu Tyr Met Arg Asn Gly Asp Phe Leu Pro Thr Arg
10     15     20     25
CTG CAG GCC CAG CAG GAT GCT GTC AAT ATA GTT TGT CAT TCA AAG ACC      267
Leu Gln Ala Gln Gln Asp Ala Val Asn Ile Val Cys His Ser Lys Thr
30     35     40

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ACC ACC AAC CCT GAG AAC AAC GTG GGC GTT ATC ACA CTG GCT AAT GAC 11
 Arg Ser Asn Pro Glu Asn Asn Val Gly Leu Ile Thr Leu Ala Asn Asp 45 55
 TGT GAA GTG CTG ACC ACA CTC ACC CCA GAC ACT GGC CGT ATC CTG TCT 163
 Cys Glu Val Leu Thr Thr Leu Thr Pro Asp Thr Gly Arg Ile Leu Ser 60 65 70
 AAG CTA CAT ACT GTC CAA CCC AAG GGC AAG ATC ACC TTC TCC ACG GGC 411
 Lys Leu His Thr Val Gln Pro Lys Gly Lys Ile Thr Phe Cys Thr Gly 75 80 85
 ATC CGC GTG GGC CAT CTG GCT CTG AAG CAC CGA CAA GGC AAG AAT CAC 454
 Ile Arg Val Ala His Leu Ala Leu Lys His Arg Gln Gly Lys Asn His 90 95 100 105
 AAG ATG CGC ATC ATT GCC TTT GTG GGA AGC CCA GTG GAG GAC AAT GAG 507
 Lys Met Arg Ile Ile Ala Phe Val Gly Ser Pro Val Glu Asp Asn Gln 110 115 120
 AAG GAT CTG GTG AAA CTG GCT AAA CGC CTC AAG AAG GAG AAA GTA AAT 555
 Lys Asp Leu Val Lys Leu Ala Lys Arg Leu Lys Lys Glu Lys Val Asn 125 130 135
 GTT GAC ATT ATC AAT TTT GGG GAA GAG GAG GTG AAC ACA GAA AAG CTG 603
 Val Asp Ile Ile Asn Phe Gly Glu Glu Glu Val Asn Thr Thr Glu Lys Leu 140 145 150
 ACA GCC TTT GTA AAC ACG TTG AAT GGC AAA GAT GGA ACC GGT TCT CAT 651
 Thr Ala Phe Val Asn Thr Leu Asn Gly Lys Asp Gly Thr Gly Ser His 155 160 165
 CTG GTG ACA GTG CCT CCT GGG CCC AGT TTG GCT GAT GCT CTC ATC AGT 699
 Leu Val Thr Val Pro Pro Gly Pro Ser Leu Ala Asp Ala Leu Ile Ser 170 175 180 185
 TCT CCG ATT TTG GCT GGT GAA GGT GGT GCC ATG CTG GGT CTT GGT GCC 747
 Ser Pro Ile Leu Ala Gly Glu Gly Gly Ala Met Leu Gly Leu Gly Ala 190 195 200
 AGT GAC TTT GAA TTT GGA GTA GAT CCC AGT GCT GAT CCT GAG CTG GCC 795
 Ser Asp Phe Glu Phe Gly Val Asp Pro Ser Ala Asp Pro Glu Leu Ala 205 210 215
 TTG GCC CTT CGT GTA TCT ATG GAA GAG CAG CGG CAG CGG CAG GAG 843
 Leu Ala Leu Arg Val Ser Met Glu Glu Gln Arg Gln Arg Gln Glu Glu 220 225 230
 GAG GCC CGG CGG GCA GCT GCA GCT TCT GCT GCT GAG GCC GGG ATT GCT 891
 Glu Ala Arg Arg Ala Ala Ala Ser Ala Ala Glu Ala Gly Ile Ala 235 240 245
 ACG ACT GGG ACT GAA GAC TCA GAC GAT GCC CTG CTG AAG ATG ACC ATC 939
 Thr Thr Gly Thr Glu Asp Ser Asp Asp Ala Leu Leu Lys Met Thr Ile 250 255 260 265
 AGC CAG CAA GAG TTT GGC CGC ACT GGG CTT CCT GAC CTA AGC AGT ATG 987
 Ser Gln Gln Glu Phe Gly Arg Thr Gly Leu Pro Asp Leu Ser Ser Met 270 275 280
 ACT GAG GAA GAG CAG ATT GCT TAT GCC ATG CAG ATG TCC CTG CAG GGA 1035
 Thr Glu Glu Glu Gln Ile Ala Tyr Ala Met Gln Met Ser Leu Gln Gly 285 290 295
 GCA GAG TTT GGC CAG GCG GAA TCA GCA GAC ATT GAT GCC AGC TCA GCT 1083
 Ala Glu Phe Gly Gln Ala Glu Ser Ala Asp Ile Asp Ala Ser Ser Ala 300 305 310
 ATG GAC ACA TCC GAG CCA GCC AAG GAG GAG GAT GAT TAC GAC GTG ATG 1131
 Met Asp Thr Ser Glu Pro Ala Lys Glu Glu Asp Asp Tyr Asp Val Met 315 320 325
 CAG GAC CCC GAG TTC CTT CAG AGT GTC CTA GAG AAC CTC CCA GGT GTG 1179
 Gln Asp Pro Glu Phe Leu Gln Ser Val Leu Glu Asn Leu Pro Gly Val 330 335 340 345
 GAT CCC AAC AAT GAA GCC ATT CGA AAT GCT ATG GGC TCC CTG GCC TCC 1227
 Asp Pro Asn Asn Glu Ala Ile Arg Asn Ala Met Gly Ser Leu Ala Ser 350 355 360
 CAG GCC ACC AAG GAC GGC AAG AAG GAC AAG AAG GAG GAA GAC AAG AAG 1275
 Gln Ala Thr Lys Asp Gly Lys Lys Asp Lys Lys Glu Glu Asp Lys Lys 365 370 375
 TGAGACTGGA GGGAAAGGGT AGCTGAGTCT GCTTAGGGAC TGCATGGGGG AATTCT 1330

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 377 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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(1x1) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

Met Val Leu Glu Ser Thr Met Val Cys Val Asp Asn Ser Glu Tyr Met
 1      5      10
Arg Asn Gly Asp Phe Leu Pro Thr Arg Leu Gln Ala Gln Gln Asp Ala
 20      30
Val Asn Ile Val Cys His Ser Lys Thr Arg Ser Asn Pro Glu Asn Asn
 35      45
Val Gly Leu Ile Thr Leu Ala Asn Asp Cys Glu Val Leu Thr Thr Leu
 50      60
Thr Pro Asp Thr Gly Arg Ile Leu Ser Lys Leu His Thr Val Gln Pro
 65      75
Lys Gly Lys Ile Thr Phe Cys Thr Gly Ile Arg Val Ala His Leu Ala
 85      95
Leu Lys His Arg Gln Gly Lys Asn His Lys Met Arg Ile Ile Ala Phe
100      110
Val Gly Ser Pro Val Glu Asp Asn Glu Lys Asp Leu Val Lys Leu Ala
115      125
Lys Arg Leu Lys Lys Glu Lys Val Asn Val Asp Ile Ile Asn Phe Gly
130      140
Glu Glu Glu Val Asn Thr Glu Lys Leu Thr Ala Phe Val Asn Thr Leu
145      155
Asn Gly Lys Asp Gly Thr Gly Ser His Leu Val Thr Val Pro Pro Gly
165      175
Pro Ser Leu Ala Asp Ala Leu Ile Ser Ser Pro Ile Leu Ala Gly Glu
180      190
Gly Gly Ala Met Leu Gly Leu Gly Ala Ser Asp Phe Glu Phe Gly Val
195      205
Asp Pro Ser Ala Asp Pro Glu Leu Ala Leu Ala Leu Arg Val Ser Met
210      220
Glu Glu Gln Arg Gln Arg Gln Glu Glu Glu Ala Arg Arg Ala Ala Ala
225      235
Ala Ser Ala Ala Glu Ala Gly Ile Ala Thr Thr Gly Thr Glu Asp Ser
245      255
Asp Asp Ala Leu Leu Lys Met Thr Ile Ser Gln Gln Glu Phe Gly Arg
260      270
Thr Gly Leu Pro Asp Leu Ser Ser Met Thr Glu Glu Glu Gln Ile Ala
275      285
Tyr Ala Met Gln Met Ser Leu Gln Gly Ala Glu Phe Gly Gln Ala Glu
290      300
Ser Ala Asp Ile Asp Ala Ser Ser Ala Met Asp Thr Ser Glu Pro Ala
305      315
Lys Glu Glu Asp Asp Tyr Asp Val Met Gln Asp Pro Glu Phe Leu Gln
325      335
Ser Val Leu Glu Asn Leu Pro Gly Val Asp Pro Asn Asn Glu Ala Ile
340      350
Arg Asn Ala Met Gly Ser Leu Ala Ser Gln Ala Thr Lys Asp Gly Lys
355      365
Lys Asp Lys Lys Glu Glu Asp Lys Lys
370      375

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(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 3841 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ix) FEATURE:
- (A) NAME/KEY: CDS
 - (B) LOCATION: 2..3121

- (ix) FEATURE:
- (A) NAME/KEY: misc feature
 - (B) LOCATION: 1..3841
 - (D) OTHER INFORMATION: /note= "GT24"

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(X1) SEQUENCE DESCRIPTION: SEQ ID NO:3:

T TCA CAG CTC CCG GCC CGA GGC ACA GAA GCC CGA GST ACG GGC CAG	40
Ser Gln Leu Pro Ala Arg Gly Thr Gln Ala Arg Xaa Thr Gly Gln	15
AGC TTC AGC CAG GGC ACG ACC AGC GGC GCC GGC CAC CTG GCG GGG CCC	94
Ser Phe Ser Gln Gly Thr Thr Ser Arg Ala Gly His Leu Ala Gly Pro	30
GAG CCC GCG CCG CCG CCG CCG CCG CCG CCG CCG GAG CCG TTC GCG CCC	142
Glu Pro Ala Pro Pro Pro Pro Pro Xaa Pro Arg Glu Pro Phe Ala Pro	45
AGC CTG GGC AGC GCC TTC CAC CTG CCC GAC GCG CCG CCC GGC GCC GCC	190
Ser Leu Gly Ser Ala Phe His Leu Pro Asp Ala Pro Ala Ala Ala	60
GCC GCC GCG CTC TAC TAC TCC ACC TCC ACG CTG CCC GCG CCG CCG CCG	238
Ala Ala Ala Leu Tyr Tyr Ser Xaa Ser Thr Leu Pro Ala Pro Pro Arg	75
GGG GGC TCC CCG CTG GCC GCG CCC CAG GGC GGT TCG CCC ACC AAG CTG	286
Gly Gly Ser Pro Leu Ala Ala Pro Gln Gly Gly Ser Pro Thr Lys Leu	95
CAG CCG GGC GGC TCG GCC CCG GAG GGC GCC ACC TAC GCC GCG CCG CCG	334
Gln Arg Gly Gly Ser Ala Pro Glu Gly Ala Thr Tyr Ala Ala Pro Arg	110
GGC TCC TCG CCC AAG CAG TCG CCC AGC CCG CTG GCC AAG TCC TAC AGC	382
Gly Ser Ser Pro Lys Gln Ser Pro Ser Arg Leu Ala Lys Ser Tyr Ser	125
ACC AGC TCG CCC ATC AAC ATC GTC GTG TCC TCG GCC GGC CTG TCC CCG	430
Thr Ser Pro Ile Asn Ile Val Val Ser Ser Ala Gly Leu Ser Pro	140
ATC CCG GTG ACC TCG CCC CCC ACC GTG CAG TCC ACC ATC TCC TCC TCG	478
Ile Arg Val Thr Ser Pro Pro Thr Val Gln Ser Thr Ile Ser Ser Ser	155
CCC ATC CAC CAG CTG AGC TCC ACC ATC GGC ACG TAC GCC ACC CTG TCG	526
Pro Ile His Gln Leu Ser Ser Thr Ile Gly Thr Tyr Ala Thr Leu Ser	175
CCC ACC AAG CCG CTG GTC CAC GCG TCC GAG CAG TAC AGC AAG CAC TCG	574
Pro Thr Lys Arg Leu Val His Ala Ser Glu Gln Tyr Ser Lys His Ser	190
CAG GAG CTG TAT GCC ACG GCC ACC CTC CAG AGG CCG GGC AGC CTG GCA	622
Gln Glu Leu Ser Ala Thr Ala Thr Leu Ser Gly Ser Leu Ala	205
GCT GGT TCC CGA GCC TCA TAC AGC AGC CAG CAT GGG CAC CTG GGC CCA	670
Ala Gly Ser Arg Ala Ser Tyr Ser Ser Gln His Gly His Leu Gly Pro	220
GAG TTG CCG GCC CTG CAG TCC CCA GAA CAC CAC ATA GAT CCC ATC TAT	718
Glu Leu Arg Ala Leu Gln Ser Pro Glu His His Ile Asp Pro Ile Tyr	235
GAA GAC CCG GTC TAT CAG AAG CCC CCT ATG AGG AGT CTC AGC CAG AGC	766
Glu Asp Arg Val Tyr Gln Lys Pro Pro Met Arg Ser Leu Ser Gln Ser	255
CAG GGG GAC CCT CTG CCG CCA GCA CAC ACC GGC ACC TAC CCG ACG AGC	814
Gln Gly Asp Pro Leu Pro Pro Ala His Thr Gly Thr Tyr Arg Thr Ser	270
ACA GCC CCA TCT TCC CCT GGT GTC GAC TCC GTC CCC TTG CAG CCG ACA	862
Thr Ala Pro Ser Ser Pro Gly Val Asp Ser Val Pro Leu Gln Arg Thr	285
GGC AGC CAG CAC GGC CCA CAG AAT GCC GCC GCG GCC ACC TTC CAG AGG	910
Gly Ser Gln His Gly Pro Gln Asn Ala Ala Ala Ala Thr Phe Gln Arg	300
GCC AGC TAT GCC GCC GGC CCA GCC TCC AAT TAC GCG GAC CCC TAC CGA	958
Ala Ser Tyr Ala Ala Gly Pro Ala Ser Asn Tyr Ala Asp Pro Tyr Arg	315
CAG CTG CAG TAT TGT CCC TCT GTT GAG TCT CCA TAC AGC AAA TCC GGC	1006
Gln Leu Gln Tyr Cys Ser Val Glu Ser Pro Tyr Ser Lys Ser Gly	335
CCT GCT CTC CCG CCT GAA GGC ACC TTG GCC AGG TCC CCG TCC ATT GAT	1054
Pro Ala Leu Pro Pro Glu Gly Thr Leu Ala Arg Ser Pro Ser Ile Asp	350
AGC ATT CAG AAA GAT CCC AGA GAA TTT GGA TGG AGA GAC CCG GAA CTG	1102
Ser Ile Gln Lys Asp Pro Arg Glu Phe Gly Trp Arg Asp Pro Gln Leu	365
CCG GAA GTG ATT CAG ATG TTG CAG CAC CAG TTT CCC TCG GTC CAG TCT	1150

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Pro	Glu	Val	Ile	Gln	Met	Leu	Gln	His	Gln	Phe	Pro	Ser	Val	Gln	Ser	
370	375	380														
AAC	GCG	GCA	GCC	TAC	TTG	CAA	CAC	CTC	TGT	TTT	GGA	GAC	AAC	AAA	ATT	1198
Asn	Ala	Ala	Ala	Tyr	Leu	Gln	His	Leu	Cys	Phe	Gly	Asp	Asn	Lys	Ile	
385	390	395														
AAA	GCC	GAG	ATA	AGG	AGA	CAA	GGA	GGC	ATC	CAG	CTC	CTG	GTG	GAC	CTG	1246
Lys	Ala	Glu	Ile	Arg	Arg	Gln	Gly	Gly	Ile	Gln	Leu	Leu	Val	Asp	Leu	
400	405	410														
TTG	GAT	CAT	CGG	ATG	ACC	GAA	GTC	CAC	CGT	AGT	GCC	TGT	GGA	GCT	CTG	1294
Leu	Asp	His	Arg	Met	Thr	Glu	Val	His	Arg	Ser	Ala	Cys	Gly	Ile	Leu	
			420						425					430		
AGA	AAC	CTG	GTG	TAT	GGG	AAG	GCC	AAC	GAT	GAT	AAC	AAA	ATT	GCC	CTG	1342
Arg	Asn	Leu	Val	Tyr	Gly	Lys	Ala	Asn	Asp	Asp	Asn	Lys	Ile	Ala	Leu	
			435					440					445			
AAA	AAC	TGT	GGT	GGC	ATC	CCA	GCA	CTG	GTG	AGG	TTA	CTC	CGC	AAG	ACG	1390
Lys	Asn	Cys	Gly	Gly	Ile	Pro	Ala	Leu	Val	Arg	Leu	Leu	Arg	Lys	Thr	
		450					455					460				
ACT	CAC	CTG	GAG	ATC	CGG	GAG	CTG	GTC	ACA	GGA	GTC	CTT	TGG	AAC	CTC	1438
Thr	Asp	Leu	Glu	Ile	Arg	Glu	Leu	Val	Thr	Gly	Val	Leu	Trp	Asn	Leu	
	465					470					475					
TCC	TCA	TGC	GAT	GCA	CTC	AAA	ATG	CCA	ATC	ATC	CAG	GAT	GCC	CTA	GCA	1486
Ser	Ser	Cys	Asp	Ala	Leu	Lys	Met	Pro	Ile	Ile	Gln	Asp	Ala	Leu	Ala	
480					485					490					495	
GTA	CTG	ACC	AAC	GCG	GTG	ATT	ATC	CCC	CAC	TCA	GGC	TGG	GAA	AAT	TCG	1534
Val	Leu	Thr	Asn	Val	Val	Ile	Ile	Pro	His	Ser	Gly	Trp	Glu	Asn	Ser	
				500					505					510		
CCT	CTT	CAG	GAT	GAT	CGG	AAA	ATA	CAG	CTG	CAT	TCA	TCA	CAG	GTG	CTG	1582
Pro	Leu	Gln	Asp	Asp	Arg	Lys	Ile	Gln	Leu	His	Ser	Ser	Gln	Val	Leu	
			515					520					525			
CGT	AAC	GCC	ACC	GGG	TGC	CTA	AGG	AAT	GTT	AGT	TCG	GCC	GGA	GAG	GAG	1630
Arg	Asn	Ala	Thr	Gly	Cys	Leu	Arg	Asn	Val	Ser	Ser	Ala	Gly	Glu	Glu	
		530					535					540				
GCC	CGC	AGA	AGG	ATG	AGA	GAG	TGT	GAT	GGG	CTT	ACG	GAT	GCC	TTG	CTG	1678
Ala	Arg	Arg	Arg	Met	Arg	Glu	Cys	Asp	Gly	Leu	Thr	Asp	Ala	Leu	Leu	
	545					550					555					
TAC	GTG	ATC	CAG	TCT	GCG	CTG	GGG	AGC	AGT	GAG	ATC	GAT	AGC	AAG	ACC	1726
Tyr	Val	Ile	Gln	Ser	Ala	Leu	Gly	Ser	Ser	Glu	Ile	Asp	Ser	Lys	Thr	
560					565					570				575		
GTT	GAA	AAC	TGT	GTG	TGC	ATT	TTA	AGG	AAC	CTC	TCG	TAC	CGG	CTG	GCG	1774
Val	Glu	Asn	Cys	Val	Cys	Ile	Leu	Arg	Asn	Leu	Ser	Tyr	Arg	Leu	Ala	
				580					585					590		
GCA	GAA	ACG	TCT	CAG	GGA	CAG	CAC	ATG	GGC	ACG	GAC	GAG	CTG	GAC	GGG	1822
Ala	Glu	Thr	Ser	Gln	Gly	Gln	His	Met	Gly	Thr	Asp	Glu	Leu	Asp	Gly	
			595					600					605			
CTA	CTC	TGT	GGC	GAG	GCC	AAT	GGC	AAG	GAT	GCT	GAG	AGC	TCT	GGG	TGC	1870
Leu	Leu	Cys	Gly	Glu	Ala	Asn	Gly	Lys	Asp	Ala	Glu	Ser	Ser	Gly	Cys	
		610					615					620				
TGG	GGC	AAG	AAG	AAG	AAG	AAA	AAG	AAA	TCC	CAA	GAT	CAG	TGG	GAT	GGA	1918
Trp	Gly	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Ser	Gln	Asp	Gln	Trp	Asp	Gly	
	625					630					635					
GTA	GGA	CCT	CTT	CCA	GAC	TGT	GCT	GAA	CCA	CCA	AAA	GGG	ATC	CAG	ATG	1966
Val	Gly	Pro	Leu	Pro	Asp	Cys	Ala	Glu	Pro	Pro	Lys	Gly	Ile	Gln	Met	
	640				645					650					655	
CTG	TGG	CAC	CCA	TCA	ATA	GTC	AAA	CCC	TAC	CTC	ACA	CTG	CTC	TCT	GAG	2014
Leu	Trp	His	Pro	Ser	Ile	Val	Lys	Pro	Tyr	Leu	Thr	Leu	Leu	Ser	Glu	
				660					665					670		
TGC	TCA	AAT	CCA	GAC	ACG	CTG	GAA	GGG	GCG	GCA	GGC	GCC	CTG	CAG	AAC	2062
Cys	Ser	Asn	Pro	Asp	Thr	Leu	Glu	Gly	Ala	Ala	Gly	Ala	Leu	Gln	Asn	
			675					680					685			
TTG	GCT	GCA	GGG	AGC	TGG	AAG	TGG	TCA	GTA	TAT	ATC	CGA	GCC	GCT	GTC	2110
Leu	Ala	Ala	Gly	Ser	Trp	Lys	Trp	Ser	Val	Tyr	Ile	Arg	Ala	Ala	Val	
		690					695					700				
CGA	AAA	GAG	AAA	GGC	CTG	CCC	ATC	CTC	GTG	GAG	CTG	CTC	CGA	ATA	GAC	2158
Arg	Lys	Glu	Lys	Gly	Leu	Pro	Ile	Leu	Val	Glu	Leu	Leu	Arg	Ile	Asp	
	705					710					715					
AAT	GAC	CGT	GTG	GTG	TGC	GCG	GTG	GCC	ACT	GCG	CTG	CGG	AAC	ATG	GCC	2206
Asn	Asp	Arg	Val	Val	Cys	Ala	Val	Ala	Thr	Ala	Leu	Arg	Asn	Met	Ala	
					725				730						735	
TTG	GAC	GTC	AGA	AAT	AAG	GAG	CTC	ATC	GGC	AAA	TAC	GCC	ATG	CGA	GAC	2254
Leu	Asp	Val	Arg	Asn	Lys	Glu	Leu	Ile	Gly	Lys	Tyr	Ala	Met	Arg	Asp	
				740					745					750		

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CTA	GTC	CAC	AGG	CTT	CCA	GGA	GGG	AAC	AAC	AGC	AAC	AAC	ACT	GCA	AGC	2382
Leu	Val	His	Arg	Leu	Pro	Gly	Gly	Asn	Asn	Ser	Asn	Asn	Thr	Ala	Ser	
			755					760					765			
AAG	GGC	ATG	TGG	GAT	GAC	ACA	GTS	ACA	GCT	GTC	TGC	TGC	ACA	CTG	CAC	2384
Lys	Ala	Met	Ser	Asp	Asp	Thr	Val	Thr	Ala	Val	Cys	Cys	Thr	Leu	His	
			770				775						780			
GAA	GTC	ATT	ACC	AAG	AAC	ATG	GAG	AAC	GCC	AAG	GCC	TTA	CGG	GAT	GCC	2398
Glu	Val	Ile	Thr	Lys	Asn	Met	Glu	Asn	Ala	Lys	Ala	Leu	Arg	Asp	Ala	
			785			790					795					
GGT	GGC	ATC	GAG	AAG	TTG	GTC	GSC	ATC	TCC	AAA	AGC	AAA	GGA	GAT	AAA	2446
Gly	Gly	Ile	Glu	Lys	Leu	Val	Gly	Ile	Ser	Lys	Ser	Lys	Gly	Asp	Lys	
					805					810					815	
CAC	TCT	CCA	AAA	GTG	GTC	AAG	GCT	GCA	TCT	CAG	GTC	CTC	AAC	AGC	ATG	2494
His	Ser	Pro	Lys	Val	Val	Lys	Ala	Ala	Ser	Gln	Val	Leu	Asn	Ser	Met	
				820					825					830		
TGG	CAG	TAC	CGA	GAT	CTG	AGG	AGT	CTC	TAC	AAA	AAG	GAT	GGA	TGG	TCA	2542
Trp	Gln	Tyr	Arg	Asp	Leu	Arg	Ser	Leu	Tyr	Lys	Lys	Asp	Gly	Trp	Ser	
			835					840					845			
CAA	TAC	CAC	TTT	GTA	GCC	TCG	TCT	TCA	ACC	ATC	GAG	AGG	GAC	CGG	CAA	2590
Gln	Tyr	His	Phe	Val	Ala	Ser	Ser	Ser	Thr	Ile	Glu	Arg	Asp	Arg	Gln	
			850				855					860				
AGG	CCC	TAC	TCC	TCC	TCC	CGC	ACG	CCC	TCC	ATC	TCC	CCT	GTG	CGC	GTG	2638
Arg	Pro	Tyr	Ser	Ser	Ser	Arg	Thr	Pro	Ser	Ile	Ser	Pro	Val	Arg	Val	
						870					875					
TCT	CCC	AAC	AAC	CGC	TCA	GCA	AGT	GCC	CCA	GCT	TCA	CCT	CGG	GAA	ATG	2686
Ser	Pro	Asn	Asn	Arg	Ser	Ala	Ser	Ala	Pro	Ala	Ser	Pro	Arg	Glu	Met	
					885					890					895	
ATC	AGC	CTC	AAA	GAA	AGG	AAA	ACA	GAC	TAC	GAG	TGC	ACC	GGC	AGC	AAC	2734
Ile	Ser	Leu	Lys	Glu	Arg	Lys	Thr	Asp	Tyr	Glu	Cys	Thr	Gly	Ser	Asn	
				900					905					910		
GCC	ACC	TAC	CAC	GGA	GGT	AAA	GGC	GAA	CAC	ACT	TCC	AGG	AAA	GAT	GCC	2782
Ala	Thr	Tyr	His	Gly	Gly	Lys	Gly	Glu	His	Thr	Ser	Arg	Lys	Asp	Ala	
			915					920					925			
ATG	ACA	GCT	CAA	AAC	ACT	GGA	ATT	TCA	ACT	TTG	TAT	AGG	AAT	TCT	TAT	2830
Met	Thr	Ala	Gln	Asn	Thr	Gly	Ile	Ser	Thr	Leu	Tyr	Arg	Asn	Ser	Tyr	
			930				935					940				
GGT	CGC	CCC	GCT	GAA	GAC	ATC	AAA	CAC	AAC	CAG	GTT	TCA	GCA	CAG	CCA	2878
Gly	Ala	Pro	Ala	Glu	Asp	Ile	Lys	His	Asn	Gln	Val	Ser	Ala	Gln	Pro	
			945			950					955					
GTC	CCA	CAG	GAG	CCC	AGC	AGA	AAA	GAT	TAC	GAG	ACC	TAC	CAG	CCA	TTT	2926
Val	Pro	Gln	Glu	Pro	Ser	Arg	Lys	Asp	Tyr	Glu	Thr	Tyr	Gln	Pro	Phe	
					965					970					975	
CAG	AAT	TCC	ACA	AGA	AAT	TAC	GAT	GAG	TCC	TTC	TTC	GAG	GAC	CAG	GTC	2974
Gln	Asn	Ser	Thr	Arg	Asn	Tyr	Asp	Glu	Ser	Phe	Phe	Glu	Asp	Gln	Val	
				980					985					990		
CAC	CAT	CGC	CCT	CCC	GCC	AGC	GAG	TAC	ACC	ATG	CAC	CTG	GGT	CTC	AAG	3022
His	His	Arg	Pro	Pro	Ala	Ser	Glu	Tyr	Thr	Met	His	Leu	Gly	Leu	Lys	
			995					1000					1005			
TCC	ACC	GGC	AAC	TAC	GTT	GAC	TTC	TAC	TCA	GCT	GCC	CGT	CCC	TAC	AGT	3070
Ser	Thr	Gly	Asn	Tyr	Val	Asp	Phe	Tyr	Ser	Ala	Ala	Ser	Pro	Tyr	Ser	
			1010				1015					1020				
GAA	CTG	AAC	TAT	GAA	ACG	AGC	CAC	TAC	CCG	GCC	TCC	CCC	GAC	TCC	TGG	3118
Glu	Leu	Asn	Tyr	Glu	Thr	Ser	His	Tyr	Pro	Ala	Ser	Pro	Asp	Ser	Trp	
			1025			1030					1035					
GTG	TGAGGAGCAG	GGCACAGGCG	CTCCGGGAAA	CAGTGCATGT	GCATGCATAC											3170
	Val															
	1040															
CACAAGACAT	TTCTTTCTGT	TTTGGTTTTT	TTCTCCTGCA	AATTTAGTTT	GTAAAGCCT											3218
GTTCATAGG	AAGGCTGTGA	TAACCACTAA	GGGAAATATT	AAGAGCTATT	TTAGAAAGCT											3266
AAATGAATCG	CAAGTTAACT	TGGAAATCAG	TAGAAAGCTA	AAGTGATCCT	AAATATGACA											3314
GTGGGCAGCA	CCTTTCCTAG	CGTGTINTGT	TAGGAGTAAC	GAGAACTGCT	TTATACTGAA											3362
CGTGGGTTGN	TTGGTAGGGT	GGAGNCGAGG	CATTCGGGCC	GGTGGGGCGT	AAGGGTTATC											3410
GTAAAGCACA	AGACACAGAA	TAGTTTACAC	ACTGTGTGGG	GGACGGCTTC	TCACGCTTTG											3458
TTTACTCTCT	TCATCCGTTG	TGACTCTAGG	CTTCAGGTTG	CATTGGGGTT	CCTCTGTACA											3506
GCAAGATGTT	TCTTGCCCTTT	TGTTAATGCA	TTGTTGTAAA	GTATTTGATG	TACATTACAG											3554
ATTAAAGAAG	NAAAGCGCGT	TGTGTATATT	ACACCAATNC	CGCCGTGTTT	CCTCATCTAT											3602
GGTTCTAAAT	ATTGCTTCAA	TTTCHAACTT	TTGAAAGATG	TATGGATTTC	CAGTTTTTCT											3650

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TTACTTTCTC CCAGTATGTT TTAACGMMN AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA
 AAAACTCGAG

1941

1941

(i) INFORMATION FOR SEQ ID NO:4:

(ii) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1040 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(iii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Ser Gln Leu Pro Ala Arg Gly Thr Gln Ala Arg Xaa Thr Gly Gln Ser
 1 5 10 15
 Phe Ser Gln Gly Thr Thr Ser Arg Ala Gly His Leu Ala Gly Pro Glu
 20 25 30
 Pro Ala Pro Pro Pro Pro Xaa Pro Arg Glu Pro Phe Ala Pro Ser
 35 40 45
 Leu Gly Ser Ala Phe His Leu Pro Asp Ala Pro Pro Ala Ala Ala Ala
 50 55 60
 Ala Ala Leu Tyr Tyr Ser Xaa Ser Thr Leu Pro Ala Pro Pro Arg Gly
 65 70 75 80
 Gly Ser Pro Leu Ala Ala Pro Gln Gly Gly Ser Pro Thr Lys Leu Gln
 85 90 95
 Arg Gly Gly Ser Ala Pro Glu Gly Ala Thr Tyr Ala Ala Pro Arg Gly
 100 105 110
 Ser Ser Pro Lys Gln Ser Pro Ser Arg Leu Ala Lys Ser Tyr Ser Thr
 115 120 125
 Ser Ser Pro Ile Asn Ile Val Ser Ser Ala Gly Leu Ser Pro Ile
 130 135 140
 Arg Val Thr Ser Pro Pro Thr Val Gln Ser Thr Ile Ser Ser Ser Pro
 145 150 155 160
 Ile His Gln Leu Ser Ser Thr Ile Gly Thr Tyr Ala Thr Leu Ser Pro
 165 170 175
 Thr Lys Arg Leu Val His Ala Ser Glu Gln Tyr Ser Lys His Ser Gln
 180 185 190
 Glu Leu Tyr Ala Thr Ala Thr Leu Gln Arg Pro Gly Ser Leu Ala Ala
 195 200 205
 Gly Ser Arg Ala Ser Tyr Ser Ser Gln His Gly His Leu Gly Pro Glu
 210 215 220
 Leu Arg Ala Leu Gln Ser Pro Glu His His Ile Asp Pro Ile Tyr Glu
 225 230 235 240
 Asp Arg Val Tyr Gln Lys Pro Pro Met Arg Ser Leu Ser Gln Ser Gln
 245 250 255
 Gly Asp Pro Leu Pro Pro Ala His Thr Gly Thr Tyr Arg Thr Ser Thr
 260 265 270
 Ala Pro Ser Ser Pro Gly Val Asp Ser Val Pro Leu Gln Arg Thr Gly
 275 280 285
 Ser Gln His Gly Pro Gln Asn Ala Ala Ala Ala Thr Phe Gln Arg Ala
 290 295 300
 Ser Tyr Ala Ala Gly Pro Ala Ser Asn Tyr Ala Asp Pro Tyr Arg Gln
 305 310 315 320
 Leu Gln Tyr Cys Pro Ser Val Glu Ser Pro Tyr Ser Lys Ser Gly Pro
 325 330 335
 Ala Leu Pro Pro Glu Gly Thr Leu Ala Arg Ser Pro Ser Ile Asp Ser
 340 345 350
 Ile Gln Lys Asp Pro Arg Glu Phe Gly Trp Arg Asp Pro Glu Leu Pro
 355 360 365
 Glu Val Ile Gln Met Leu Gln His Gln Phe Pro Ser Val Gln Ser Asn
 370 375 380
 Ala Ala Ala Tyr Leu Gln His Leu Cys Phe Gly Asp Asn Lys Ile Lys
 385 390 395 400
 Ala Glu Ile Arg Arg Gln Gly Gly Ile Gln Leu Leu Val Asp Leu Leu
 405 410 415

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Asp His Arg Met Thr Glu Val His Arg Ser Ala Cys Gly Ala Leu Arg
 420 425 430
 Asn Leu Val Tyr Gly Lys Ala Asn Asp Asp Asn Lys Ile Ala Leu Lys
 435 440 445
 Asn Cys Gly Gly Ile Pro Ala Leu Val Arg Leu Leu Arg Lys Thr Thr
 450 455 460
 Asp Leu Glu Ile Arg Gly Leu Val Thr Gly Val Leu Trp Asn Leu Ser
 465 470 475 480
 Ser Cys Asp Ala Leu Lys Met Pro Ile Ile Gln Asp Ala Leu Ala Val
 485 490 495
 Leu Thr Asn Ala Val Ile Ile Pro His Ser Gly Trp Glu Asn Ser Pro
 500 505 510
 Leu Gln Asp Asp Arg Lys Ile Gln Leu His Ser Ser Gln Val Leu Arg
 515 520 525
 Asn Ala Thr Gly Cys Leu Arg Asn Val Ser Ser Ala Gly Glu Glu Ala
 530 535 540
 Arg Arg Arg Met Arg Glu Cys Asp Gly Leu Thr Asp Ala Leu Leu Tyr
 545 550 555 560
 Val Ile Gln Ser Ala Leu Gly Ser Ser Glu Ile Asp Ser Lys Thr Val
 565 570 575
 Glu Asn Cys Val Cys Ile Leu Arg Asn Leu Ser Tyr Arg Leu Ala Ala
 580 585 590
 Glu Thr Ser Gln Gly Gln His Met Gly Thr Asp Glu Leu Asp Gly Leu
 595 600 605
 Leu Cys Gly Glu Ala Asn Gly Lys Asp Ala Glu Ser Ser Gly Cys Trp
 610 615 620
 Gly Lys Lys Lys Lys Lys Lys Ser Gln Asp Gln Trp Asp Gly Val
 625 630 635 640
 Gly Pro Leu Pro Asp Cys Ala Glu Pro Pro Lys Gly Ile Gln Met Leu
 645 650 655
 Trp His Pro Ser Ile Val Lys Pro Tyr Leu Thr Leu Leu Ser Glu Cys
 660 665 670
 Ser Asn Pro Asp Thr Leu Glu Gly Ala Ala Gly Ala Leu Gln Asn Leu
 675 680 685
 Ala Ala Gly Ser Trp Lys Trp Ser Val Tyr Ile Arg Ala Ala Val Arg
 690 695 700
 Lys Glu Lys Gly Leu Pro Ile Leu Val Glu Leu Leu Arg Ile Asp Asn
 705 710 715 720
 Asp Arg Val Val Cys Ala Val Ala Thr Ala Leu Arg Asn Met Ala Leu
 725 730 735
 Asp Val Arg Asn Lys Glu Leu Ile Gly Lys Tyr Ala Met Arg Asp Leu
 740 745 750
 Val His Arg Leu Pro Gly Gly Asn Asn Ser Asn Asn Thr Ala Ser Lys
 755 760 765
 Ala Met Ser Asp Asp Thr Val Thr Ala Val Cys Cys Thr Leu His Glu
 770 775 780
 Val Ile Thr Lys Asn Met Glu Asn Ala Lys Ala Leu Arg Asp Ala Gly
 785 790 795 800
 Gly Ile Glu Lys Leu Val Gly Ile Ser Lys Ser Lys Gly Asp Lys His
 805 810 815
 Ser Pro Lys Val Val Lys Ala Ala Ser Gln Val Leu Asn Ser Met Trp
 820 825 830
 Gln Tyr Arg Asp Leu Arg Ser Leu Tyr Lys Lys Asp Gly Trp Ser Gln
 835 840 845
 Tyr His Phe Val Ala Ser Ser Ser Thr Ile Glu Arg Asp Arg Gln Arg
 850 855 860
 Pro Tyr Ser Ser Ser Arg Thr Pro Ser Ile Ser Pro Val Arg Val Ser
 865 870 875 880
 Pro Asn Asn Arg Ser Ala Ser Ala Pro Ala Ser Pro Arg Glu Met Ile
 885 890 895
 Ser Leu Lys Glu Arg Lys Thr Asp Tyr Glu Cys Thr Gly Ser Asn Ala
 900 905 910
 Thr Tyr His Gly Gly Lys Gly Glu His Thr Ser Arg Lys Asp Ala Met
 915 920 925

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Thr Ala Gln Asn Thr Gly Ile Ser Thr Leu Tyr Arg Asn Ser Tyr Gly
 930 935 940
 Ala Pro Ala Glu Asp Ile Lys His Asn Gln Val Ser Ala Gln Pro Val
 945 950 955
 Pro Gln Glu Pro Ser Arg Lys Asp Tyr Glu Thr Tyr Gln Pro Phe Gln
 965 970 975
 Asn Ser Thr Arg Asn Tyr Asp Glu Ser Phe Phe Glu Asp Gln Val His
 980 985 990
 His Arg Pro Pro Ala Ser Glu Tyr Thr Met His Leu Gly Leu Lys Ser
 995 1000 1005
 Thr Gly Asn Tyr Val Asp Phe Tyr Ser Ala Ala Arg Pro Tyr Ser Glu
 1010 1015 1020
 Leu Asn Tyr Glu Thr Ser His Tyr Pro Ala Ser Pro Asp Ser Trp Val
 1025 1030 1035 1040

(2) INFORMATION FOR SEQ ID NO:5:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 3907 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 142..3777
 (D) OTHER INFORMATION: /note= "p0071"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTACTGTTGT TTTTGAGGGG CGGGCAGCCG CGCCGCCGCG GCACTTTTTT AATTTTTTCG	60
GGTGCCGCAG CAGCGACCCC TCGGCGCCGA TGTCCTTGAT CCCTGGAGCG ACGACGGCCC	120
CTGCCTAAGC TGGGAAGAGG A ATG CCA GCT CCT GAG CAG GCC TCA TTG GTG Met Pro Ala Pro Glu Gln Ala Ser Leu Val	171
GAG GAG GGG CAA CCA CAG ACC CGC CAG GAA GCT GCC TCC ACT GGC CCA Glu Glu Gly Gln Pro Gln Thr Arg Gln Glu Ala Ala Ser Thr Gly Pro	219
GGC ATG GAA CCC GAG ACC ACA GCC ACC ACT ATT CTA GCA TCC GTG AAG Gly Met Glu Pro Glu Thr Thr Ala Thr Thr Ile Leu Ala Ser Val Lys	267
GAG CAG GAG CTT CAG TTT CAG CGA CTC ACC CGA GAA CTG GAA GTG GAA Glu Gln Glu Leu Gln Phe Gln Arg Leu Thr Arg Glu Leu Glu Val Glu	315
AGG CAG ATT GTT GCC AGT CAG CTA GAA AGA TGT AGG CTT GGA GCA GAA Arg Gln Ile Val Ala Ser Gln Leu Glu Arg Cys Arg Leu Gly Ala Glu	363
TCA CCA AGC ATC GCC AGC ACC AGC TCA ACT GAG AAG TCA TTT CCT TGG Ser Pro Ser Ile Ala Ser Thr Ser Ser Thr Glu Lys Ser Phe Pro Trp	411
AGA TCA ACA GAC GTG CCA AAT ACT GGT GTA AGC AAA CCT AGA GTT TCT Arg Ser Thr Asp Val Pro Asn Thr Gly Val Ser Lys Pro Arg Val Ser	459
GAC GCT GTC CAG CCC AAC AAC TAT CTC ATC AGG ACA GAG CCA GAA CAA Asp Ala Val Gln Pro Asn Asn Tyr Leu Ile Arg Thr Glu Pro Glu Gln	507
GGA ACC CTC TAT TCA CCA GAA CAG ACA TCT CTC CAT GAA AGT GAG GGA Gly Thr Leu Tyr Ser Pro Glu Gln Thr Ser Leu His Glu Ser Glu Gly	555
TCA TTG GGT AAC TCA AGA AGT TCA ACA CAA ATG AAT TCT TAT TCC GAC Ser Leu Gly Asn Ser Arg Ser Ser Thr Gln Met Asn Ser Tyr Ser Asp	603
AGT GGA TAC CAG GAA GCA GGG AGT TTC CAC AAC AGC CAG AAC GTG AGC Ser Gly Tyr Gln Glu Ala Gly Ser Phe His Asn Ser Gln Asn Val Ser	651
AAG GCA GAC AAC AGA CAG CAG CAT TCA TTC ATA GGA TCA ACT AAC AAC Lys Ala Asp Asn Arg Gln Gln His Ser Phe Ile Gly Ser Thr Asn Asn	699
CAT GTG GTG AGG AAT TCA AGA GCT GAA GGA CAA ACA CTG GTT CAG CCA His Val Val Arg Asn Ser Arg Ala Glu Gly Gln Thr Leu Val Gln Pro	747

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190						195					200					
TCA Ser	GTA Val	GCC Ala 205	AAT Asn	CGG Arg	GCC Ala	ATG Met	AGA Arg 210	AGA Arg	GTT Val	AGT Ser	TCA Ser	GTT Val 215	GCA Pro	TCT Ser	AGA Arg	705
GCA Ala	CAG Gln 220	TCT Ser	CCT Pro	TCT Ser	TAT Tyr	GTT Val 225	ATC Ile	AGC Ser	ACA Thr	GGC Gly	GTG Val 230	TCT Ser	CCT Pro	TCA Ser	AGG Arg	843
GGG Gly 235	TCT Ser	CTG Leu	AGA Arg	ACT Thr	TCT Ser 240	CTG Leu	GGT Gly	AGT Ser	GGA Gly	TTT Phe 245	GGC Gly	TCT Ser	CCG Pro	TCA Ser	GTG Val 250	891
ACC Thr	GAC Asp	CCC Pro	CGA Arg	CCT Pro 255	CTG Leu	AAC Asn	CCC Pro	AGT Ser	GCA Ala 260	TAT Tyr	TCC Ser	TCC Ser	ACC Thr	ACA Thr 265	TTA Leu	939
CCT Pro	GCT Ala	GCA Ala	CGG Arg 270	GCA Ala	GCC Ala	TCT Ser	CCG Pro	TAC Tyr 275	TCA Ser	CAG Gln	AGA Arg	CCC Pro	GCC Ala 280	TCC Ser	CCA Pro	987
ACA Thr	GCT Ala	ATA Ile 285	CGG Arg	CGG Arg	ATT Ile	GGG Gly	TCA Ser 290	GTC Val	ACC Thr	TCC Ser	CGG Arg	CAG Gln 295	ACC Thr	TCC Ser	AAT Asn	1035
CCC Pro	AAC Asn 300	GGA Gly	CCA Pro	ACC Thr	CCT Pro	CAA Gln 305	TAC Tyr	CAA Gln	ACC Thr	ACC Thr	GCC Ala 310	AGA Arg	GTG Val	GGG Gly	TCC Ser	1083
CCA Pro 315	CTG Leu	ACC Thr	CTG Leu	ACG Thr	GAT Asp 320	GCA Ala	CAG Gln	ACT Thr	CGA Arg	GTA Val 325	GCT Ala	TCC Ser	CCA Pro	TCC Ser	CAA Gln 330	1131
GGC Gly	CAG Gln	GTG Val	GGG Gly	TCG Ser 335	TCG Ser	TCC Ser	CCC Pro	AAA Lys	CGC Arg 340	TCA Ser	GGG Gly	ATG Met	ACC Thr	GCC Ala 345	GTA Val	1179
CCA Pro	CAG Gln	CAT His 350	CTG Leu	GGA Gly	CCT Pro	TCA Ser	CTG Leu	CAA Gln 355	AGG Arg	ACT Thr	GTT Val	CAT His	GAC Asp 360	ATG Met	GAG Glu	1227
CAA Gln	TTG Phe	GGA Gly 365	CAG Gln	CAG Gln	CAG Gln	TAT Tyr	GAC Asp 370	ATT Ile	TAT Tyr	GAG Glu	AGG Arg	ATG Met 375	GTT Val	CCA Pro	CCC Pro	1275
AGG Arg	CCA Pro 380	GAC Asp	AGC Ser	CTG Leu	ACA Thr	GGC Gly 385	TTA Leu	CGG Arg	AGT Ser	TCC Ser	TAT Tyr 390	GCT Ala	AGT Ser	CAG Gln	CAT His	1323
AGT Ser 395	CAG Gln	CTT Leu	GGG Gly	CAA Gln	GAC Asp 400	CTT Leu	CGT Arg	TCT Ser	GCC Ala	GTG Val 405	TCT Ser	CCC Pro	GAC Asp	TTG Leu	CAC His 410	1371
ATT Ile	ACT Thr	CCT Pro	ATA Ile	TAT Tyr 415	GAG Glu	GGG Gly	AGG Arg	ACC Thr	TAT Tyr 420	TAC Tyr	AGC Ser	CCA Pro	GTG Val	TAC Tyr 425	CGC Arg	1419
AGC Ser	CCA Pro	AAC Asn	CAT His 430	GGA Gly	ACT Thr	GTG Val	GAG Glu	CTC Leu 435	CAA Gln	GGA Gly	TCG Ser	CAG Gln	ACG Thr 440	GCG Ala	TTG Leu	1467
TAT Tyr	CGC Arg	ACA Thr 445	GGT Gly	GTA Val	TCA Ser	GGT Gly	ATT Ile 450	GGA Gly	AAT Asn	CTA Leu	CAA Gln 455	AGG Arg	ACA Thr	TCC Ser	AGC Ser	1515
CAA Gln	CGA Arg 460	AGT Ser	ACC Thr	CTT Leu	ACA Thr	TAC Tyr 465	CAA Gln	AGA Arg	AAT Asn	AAT Asn	TAT Tyr 470	GCT Ala	CTG Leu	AAC Asn	ACA Thr	1563
ACA Thr 475	GCT Ala	ACC Thr	TAC Tyr	GCG Ala	GAG Glu 480	CCC Pro	TAC Tyr	AGG Arg	CCT Pro	ATA Ile 485	CAA Gln	TAC Tyr	CGA Arg	GTG Val	CAA Gln 490	1611
GAG Glu	TGC Cys	AAT Asn	TAT Tyr	AAC Asn 495	AGG Arg	CTT Leu	CAG Gln	CAT His	GCA Ala 500	GTG Val	CCG Pro	GCT Ala	GAT Asp	GAT Asp 505	GGC Gly	1659
ACC Thr	ACA Thr	AGA Arg	TCC Ser 510	CCA Pro	TCA Ser	ATA Ile	GAC Asp	AGC Ser 515	ATT Ile	CAG Gln	AAG Lys	GAC Asp	CCC Pro 520	AGG Arg	GAG Glu	1707
TTT Phe	GCC Ala	TGG Trp 525	CGT Arg	GAT Asp	CCT Pro	GAG Glu	TTG Leu 530	CCT Pro	GAG Glu	GTC Val	ATT Ile	CAC His 535	ATG Met	CTT Leu	GAG Glu	1755
CAC His	CAG Gln 540	TTG Phe	CCA Pro	TCT Ser	GTT Val	CAG Gln 545	GCA Ala	AAT Asn	GCA Ala	ACG Ala 550	GCC Ala	TAC Tyr	CTG Leu	CAG Gln	CAC His	1803
CTG Leu 555	TGC Cys	TTT Phe	GGT Gly	GAC Asp	AAC Asn 560	AAA Lys	GTG Val	AAG Lys	ATG Met	GAG Gln 565	GTG Val	TGT Cys	AGG Arg	TTA Leu	GGG Gly 570	1851
GCA Ala	ATC Met	AAG Lys	CAT His	CTG Leu	GTT Val	GAC Asp	CTT Leu	CTC Leu	CAC His	AGA Arg	GTT Val	TTG Leu	GAA Arg	GTT Val		1899

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Gly	Ile	Lys	His	Leu	Val	Asp	Leu	Leu	Asp	His	Arg	Val	Leu	Glu	Val	
				575					580					585		
CAG	AAG	AAT	GCT	TGT	GGT	GCC	CTT	CGA	AAC	CTC	GTT	TTT	GGC	AAG	TCT	1943
Gln	Lys	Asn	Ala	Cys	Gly	Ala	Leu	Arg	Asn	Leu	Val	Phe	Gly	Lys	Ser	
			590					595					600			
ACA	GAT	GAA	AAT	AAA	ATA	GCA	ATG	AAG	AAT	GTT	GGT	GGG	ATA	CCT	GCC	1995
Thr	Asp	Glu	Asn	Lys	Ile	Ala	Met	Lys	Asn	Val	Gly	Gly	Ile	Pro	Ala	
		605					610					615				
TTG	TTG	CGA	CTG	TTG	AGA	AAA	TCT	ATT	GAT	GCA	GAA	GTA	AGG	GAG	CTT	2043
Leu	Leu	Arg	Leu	Leu	Arg	Lys	Ser	Ile	Asp	Ala	Glu	Val	Arg	Glu	Leu	
	620					625					630					
GTT	ACA	GGA	GTT	CTT	TGG	AAT	TTA	TCC	TCA	TGT	GAT	GCT	GTA	AAA	ATG	2091
Val	Thr	Gly	Val	Leu	Trp	Asn	Leu	Ser	Ser	Cys	Asp	Ala	Val	Lys	Met	
	635				640					645					650	
ACA	ATC	ATT	CGA	GAT	GCT	CTC	TCA	ACC	TTA	ACA	AAC	ACT	GTG	ATT	GTT	2139
Thr	Ile	Ile	Arg	Asp	Ala	Leu	Ser	Thr	Leu	Thr	Asn	Thr	Val	Ile	Val	
			655						660					665		
CCA	CAT	TCT	GGA	TGG	AAT	AAC	TCT	TCT	TTT	GAT	GAT	GAT	CAT	AAA	ATT	2187
Pro	His	Ser	Gly	Trp	Asn	Asn	Ser	Ser	Phe	Asp	Asp	Asp	His	Lys	Ile	
			670					675					680			
AAA	TTT	CAG	ACT	TCA	CTA	GTT	CTG	CGT	AAC	ACG	ACA	GGT	TGC	CTA	AGG	2235
Lys	Phe	Gln	Thr	Ser	Leu	Val	Leu	Arg	Asn	Thr	Thr	Gly	Cys	Leu	Arg	
		685					690					695				
AAC	CTC	ACG	TCC	GCG	GGG	GAA	GAA	GCT	CGG	AAG	CAA	ATG	CGG	TCC	TGC	2283
Asn	Leu	Thr	Ser	Ala	Gly	Glu	Glu	Ala	Arg	Lys	Gln	Met	Arg	Ser	Cys	
	700					705					710					
GAG	GGG	CTG	GTA	GAC	TCA	CTG	TTG	TAT	GTG	ATC	CAC	ACG	TGT	GTG	AAC	2331
Glu	Gly	Leu	Val	Asp	Ser	Leu	Leu	Tyr	Val	Ile	His	Thr	Cys	Val	Asn	
	715				720				725						730	
ACA	TCC	GAT	TAC	GAC	AGC	AAG	ACG	GTG	GAG	AAC	TGC	GTG	TGC	ACC	CTG	2379
Thr	Ser	Asp	Tyr	Asp	Ser	Lys	Thr	Val	Glu	Asn	Cys	Val	Cys	Thr	Leu	
			735					740					745			
AGG	AAC	CTG	TCC	TAT	CGG	CTG	GAG	CTG	GAG	GTG	CCC	CAG	GCC	CGG	TTA	2427
Arg	Asn	Leu	Ser	Tyr	Arg	Leu	Glu	Leu	Glu	Val	Pro	Gln	Ala	Arg	Leu	
			750					755					760			
CTG	GGA	CTG	AAC	GAA	TTG	GAT	GAC	TTA	CTA	GGA	AAA	GAG	TCT	CCC	AGC	2475
Leu	Gly	Leu	Asn	Glu	Leu	Asp	Asp	Leu	Leu	Gly	Lys	Glu	Ser	Pro	Ser	
		765					770					775				
AAA	GAC	TCT	GAG	CCA	AGT	TGC	TGG	GGG	AAG	AAG	AAG	AAA	AAG	AAA	AAG	2523
Lys	Asp	Ser	Glu	Pro	Ser	Cys	Trp	Gly	Lys	Lys	Lys	Lys	Lys	Lys	Lys	
	780					785					790					
AGG	ACT	CCG	CAA	GAA	GAT	CAA	TGG	GAT	GGA	GTT	GGT	CCT	ATC	CCA	GGA	2571
Arg	Thr	Pro	Gln	Glu	Asp	Gln	Trp	Asp	Gly	Val	Gly	Pro	Ile	Pro	Gly	
	795				800				805						810	
CTG	TCG	AAG	TCC	CCC	AAA	GGG	GTT	GAG	ATG	CTG	TGG	CAC	CCA	TCG	GTG	2619
Leu	Ser	Lys	Ser	Pro	Lys	Gly	Val	Glu	Met	Leu	Trp	His	Pro	Ser	Val	
			815						820					825		
GTA	AAA	CCA	TAT	CTG	ACT	CTT	CTA	GCA	GAA	AGT	TCC	AAC	CCA	GCC	ACC	2667
Val	Lys	Pro	Tyr	Leu	Thr	Leu	Leu	Ala	Glu	Ser	Ser	Asn	Pro	Ala	Thr	
			830					835					840			
TTG	GAA	GGC	TCT	GCA	GGG	TCT	CTC	CAG	AAC	CTC	TCT	GCT	AGC	AAC	TGG	2715
Leu	Glu	Gly	Ser	Ala	Gly	Ser	Leu	Gln	Asn	Leu	Ser	Ala	Ser	Asn	Trp	
		845					850					855				
AAG	TTT	GCA	TAT	ATC	CGG	GGC	GGC	CGT	CCG	AAA	AGA	AAA	GGG	CTC		2763
Lys	Phe	Ala	Ala	Tyr	Ile	Arg	Gly	Gly	Arg	Pro	Lys	Arg	Lys	Gly	Leu	
	860				865					870						
CCC	ATC	CTT	GTG	GAG	CTT	CTG	AGA	ATG	GAT	AAC	GAT	AGA	GTT	GTT	TCT	2811
Pro	Ile	Leu	Val	Glu	Leu	Leu	Arg	Met	Asp	Asn	Asp	Arg	Val	Val	Ser	
	875				880				885						890	
TCC	GGT	GCA	ACA	GCC	TTG	AGG	AAT	ATG	GCA	CTA	GAT	GTT	CGC	AAC	AAG	2859
Ser	Gly	Ala	Thr	Ala	Leu	Arg	Asn	Met	Ala	Leu	Asp	Val	Arg	Asn	Lys	
			895					900						905		
GAG	CTC	ATA	GGC	AAA	TAC	GCC	ATG	CGA	GAC	CTG	GTC	AAC	CGG	CTC	CCC	2907
Glu	Leu	Ile	Gly	Lys	Tyr	Ala	Met	Arg	Asp	Leu	Val	Asn	Arg	Leu	Pro	
		910						915					920			
GGC	GGC	AAT	GGC	CCC	AGT	GTC	TTG	TCT	GAT	GAG	ACC	ATG	GCA	GCC	ATC	2955
Gly	Gly	Asn	Gly	Pro	Ser	Val	Leu	Ser	Asp	Glu	Thr	Met	Ala	Ala	Ile	
		925					930					935				
TGC	TGT	GCT	CTG	CAC	GAG	GTC	ACC	AGT	AAA	AAC	ATG	GAG	AAC	GCA	AAA	3003
Cys	Cys	Ala	Leu	His	Glu	Val	Thr	Ser	Lys	Asn	Met	Glu	Asn	Ala	Lys	
	940					945					950					

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GCC CTG GCC GAC TCA GGA GGC ATA GAG AAG CTG GTG AAC ATA ACC AAA Ala Leu Ala Asp Ser Gly Gly Ile Glu Lys Leu Val Asn Ile Thr Lys 955 960 965 970 975 980 985 990 995 1000 1005 1010 1015 1020 1025 1030 1035 1040 1045 1050 1055 1060 1065 1070 1075 1080 1085 1090 1095 1100 1105 1110 1115 1120 1125 1130 1135 1140 1145 1150 1155 1160 1165 1170 1175 1180 1185 1190 1195 1200 1205 1210 1215 1220 1225 1230 1235 1240 1245 1250 1255 1260 1265 1270 1275 1280 1285 1290 1295 1300 1305 1310 1315 1320 1325 1330 1335 1340 1345 1350 1355 1360 1365 1370 1375 1380 1385 1390 1395 1400 1405 1410 1415 1420 1425 1430 1435 1440 1445 1450 1455 1460 1465 1470 1475 1480 1485 1490 1495 1500 1505 1510 1515 1520 1525 1530 1535 1540 1545 1550 1555 1560 1565 1570 1575 1580 1585 1590 1595 1600 1605 1610 1615 1620 1625 1630 1635 1640 1645 1650 1655 1660 1665 1670 1675 1680 1685 1690 1695 1700 1705 1710 1715 1720 1725 1730 1735 1740 1745 1750 1755 1760 1765 1770 1775 1780 1785 1790 1795 1800 1805 1810 1815 1820 1825 1830 1835 1840 1845 1850 1855 1860 1865 1870 1875 1880 1885 1890 1895 1900 1905 1910 1915 1920 1925 1930 1935 1940 1945 1950 1955 1960 1965 1970 1975 1980 1985 1990 1995 2000 2005 2010 2015 2020 2025 2030 2035 2040 2045 2050 2055 2060 2065 2070 2075 2080 2085 2090 2095 2100 2105 2110 2115 2120 2125 2130 2135 2140 2145 2150 2155 2160 2165 2170 2175 2180 2185 2190 2195 2200 2205 2210 2215 2220 2225 2230 2235 2240 2245 2250 2255 2260 2265 2270 2275 2280 2285 2290 2295 2300 2305 2310 2315 2320 2325 2330 2335 2340 2345 2350 2355 2360 2365 2370 2375 2380 2385 2390 2395 2400 2405 2410 2415 2420 2425 2430 2435 2440 2445 2450 2455 2460 2465 2470 2475 2480 2485 2490 2495 2500 2505 2510 2515 2520 2525 2530 2535 2540 2545 2550 2555 2560 2565 2570 2575 2580 2585 2590 2595 2600 2605 2610 2615 2620 2625 2630 2635 2640 2645 2650 2655 2660 2665 2670 2675 2680 2685 2690 2695 2700 2705 2710 2715 2720 2725 2730 2735 2740 2745 2750 2755 2760 2765 2770 2775 2780 2785 2790 2795 2800 2805 2810 2815 2820 2825 2830 2835 2840 2845 2850 2855 2860 2865 2870 2875 2880 2885 2890 2895 2900 2905 2910 2915 2920 2925 2930 2935 2940 2945 2950 2955 2960 2965 2970 2975 2980 2985 2990 2995 3000 3005 3010 3015 3020 3025 3030 3035 3040 3045 3050 3055 3060 3065 3070 3075 3080 3085 3090 3095 3100 3105 3110 3115 3120 3125 3130 3135 3140 3145 3150 3155 3160 3165 3170 3175 3180 3185 3190 3195 3200 3205 3210 3215 3220 3225 3230 3235 3240 3245 3250 3255 3260 3265 3270 3275 3280 3285 3290 3295 3300 3305 3310 3315 3320 3325 3330 3335 3340 3345 3350 3355 3360 3365 3370 3375 3380 3385 3390 3395 3400 3405 3410 3415 3420 3425 3430 3435 3440 3445 3450 3455 3460 3465 3470 3475 3480 3485 3490 3495 3500 3505 3510 3515 3520 3525 3530 3535 3540 3545 3550 3555 3560 3565 3570 3575 3580 3585 3590 3595 3600 3605 3610 3615 3620 3625 3630 3635 3640 3645 3650 3655 3660 3665 3670 3675 3680 3685 3690 3695 3700 3705 3710 3715 3720 3725 3730 3735 3740 3745 3750 3755 3760 3765 3770 3775 3780 3785 3790 3795 3800 3805 3810 3815 3820 3825 3830 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5835 5840 5845 5850 5855 5860 5865 5870 5875 5880 5885 5890 5895 5900 5905 5910 5915 5920 5925 5930 5935 5940 5945 5950 5955 5960 5965 5970 5975 5980 5985 5990 5995 6000 6005 6010 6015 6020 6025 6030 6035 6040 6045 6050 6055 6060 6065 6070 6075 6080 6085 6090 6095 6100 6105 6110 6115 6120 6125 6130 6135 6140 6145 6150 6155 6160 6165 6170 6175 6180 6185 6190 6195 6200 6205 6210 6215 6220 6225 6230 6235 6240 6245 6250 6255 6260 6265 6270 6275 6280 6285 6290 6295 6300 6305 6310 6315 6320 6325 6330 6335 6340 6345 6350 6355 6360 6365 6370 6375 6380 6385 6390 6395 6400 6405 6410 6415 6420 6425 6430 6435 6440 6445 6450 6455 6460 6465 6470 6475 6480 6485 6490 6495 6500 6505 6510 6515 6520 6525 6530 6535 6540 6545 6550 6555 6560 6565 6570 6575 6580 6585 6590 6595 6600 6605 6610 6615 6620 6625 6630 6635 6640 6645 6650 6655 6660 6665 6670 6675 6680 6685 6690 6695 6700 6705 6710 6715 6720 6725 6730 6735 6740 6745 6750 6755 6760 6765 6770 6775 6780 6785 6790 6795 6800 6805 6810 6815 6820 6825 6830 6835 6840 6845 6850 6855 6860 6865 6870 6875 6880 6885 6890 6895 6900 6905 6910 6915 6920 6925 6930 6935 6940 6945 6950 6955 6960 6965 6970 6975 6980 6985 6990 6995 7000 7005 7010 7015 7020 7025 7030 7035 7040 7045 7050 7055 7060 7065 7070 7075 7080 7085 7090 7095 7100 7105 7110 7115 7120 7125 7130 7135 7140 7145 7150 7155 7160 7165 7170 7175 7180 7185 7190 7195 7200 7205 7210 7215 7220 7225 7230 7235 7240 7245 7250 7255 7260 7265 7270 7275 7280 7285 7290 7295 7300 7305 7310 7315 7320 7325 7330 7335 7340 7345 7350 7355 7360 7365 7370 7375 7380 7385 7390 7395 7400 7405 7410 7415 7420 7425 7430 7435 7440 7445 7450 7455 7460 7465 7470 7475 7480 7485 7490 7495 7500 7505 7510 7515 7520 7525 7530 7535 7540 7545 7550 7555 7560 7565 7570 7575 7580 7585 7590 7595 7600 7605 7610 7615 7620 7625 7630 7635 7640 7645 7650 7655 7660 7665 7670 7675 7680 7685 7690 7695 7700 7705 7710 7715 7720 7725 7730 7735 7740 7745 7750 7755 7760 7765 7770 7775 7780 7785 7790 7795 7800 7805 7810 7815 7820 7825 7830 7835 7840 7845 7850 7855 7860 7865 7870 7875 7880 7885 7890 7895 7900 7905 7910 7915 7920 7925 7930 7935 7940 7945 7950 7955 7960 7965 7970 7975 7980 7985 7990 7995 8000 8005 8010 8015 8020 8025 8030 8035 8040 8045 8050 8055 8060 8065 8070 8075 8080 8085 8090 8095 8100 8105 8110 8115 8120 8125 8130 8135 8140 8145 8150 8155 8160 8165 8170 8175 8180 8185 8190 8195 8200 8205 8210 8215 8220 8225 8230 8235 8240 8245 8250 8255 8260 8265 8270 8275 8280 8285 8290 8295 8300 8305 8310 8315 8320 8325 8330 8335 8340 8345 8350 8355 8360 8365 8370 8375 8380 8385 8390 8395 8400 8405 8410 8415 8420 8425 8430 8435 8440 8445 8450 8455 8460 8465 8470 8475 8480 8485 8490 8495 8500 8505 8510 8515 8520 8525 8530 8535 8540 8545 8550 8555 8560 8565 8570 8575 8580 8585 8590 8595 8600 8605 8610 8615 8620 8625 8630 8635 8640 8645 8650 8655 8660 8665 8670 8675 8680 8685 8690 8695 8700 8705 8710 8715 8720 8725 8730 8735 8740 8745 8750 8755 8760 8765 8770 8775 8780 8785 8790 8795 8800 8805 8810 8815 8820 8825 8830 8835 8840 8845 8850 8855 8860 8865 8870 8875 8880 8885 8890 8895 8900 8905 8910 8915 8920 8925 8930 8935 8940 8945 8950 8955 8960 8965 8970 8975 8980 8985 8990 8995 9000 9005 9010 9015 9020 9025 9030 9035 9040 9045 9050 9055 9060 9065 9070 9075 9080 9085 9090 9095 9100 9105 9110 9115 9120 9125 9130 9135 9140 9145 9150 9155 9160 9165 9170 9175 9180 9185 9190 9195 9200 9205 9210 9215 9220 9225 9230 9235 9240 9245 9250 9255 9260 9265 9270 9275 9280 9285 9290 9295 9300 9305 9310 9315 9320 9325 9330 9335 9340 9345 9350 9355 9360 9365 9370 9375 9380 9385 9390 9395 9400 9405 9410 9415 9420 9425 9430 9435 9440 9445 9450 9455 9460 9465 9470 9475 9480 9485 9490 9495 9500 9505 9510 9515 9520 9525 9530 9535 9540 9545 9550 9555 9560 9565 9570 9575 9580 9585 9590 9595 9600 9605 9610 9615 9620 9625 9630 9635 9640 9645 9650 9655 9660 9665 9670 9675 9680 9685 9690 9695 9700 9705 9710 9715 9720 9725 9730 9735 9740 9745 9750 9755 9760 9765 9770 9775 9780 9785 9790 9795 9800 9805 9810 9815 9820 9825 9830 9835 9840 9845 9850 9855 9860 9865 9870 9875 9880 9885 9890 9895 9900 9905 9910 9915 9920 9925 9930 9935 9940 9945 9950 9955 9960 9965 9970 9975 9980 9985 9990 9995 10000 10005 10010 10015 10020 10025 10030 10035 10040 10045 10050 10055 10060 10065 10070 10075 10080 10085 10090 10095 10100 10105 10110 10115 10120 10125 10130 10135 10140 10145 10150 10155 10160 10165 10170 10175 10180 10185 10190 10195 10200 10205 10210 10215 10220 10225 10230 10235 10240 10245 10250 10255 10260 10265 10270 10275 10280 10285 10290 10295 10300 10305 10310 10315 10320 10325 10330 10335 10340 10345 10350 10355 10360 10365 10370 10375 10380 10385 10390 10395 10400 10405 10410 10415 10420 10425 10430 10435 10440 10445 10450 10455 10460 10465 10470 10475 10480 10485 10490 10495 10500 10505 10510 10515 10520 10525 10530 10535 10540 10545 10550 10555 10560 10565 10570 10575 10580 10585 10590 10595 10600 10605 10610 10615 10620 10625 10630 10635 10640 10645 10650 10655 10660 10665 10670 10675 10680 10685 10690 10695 10700 10705 10710 10715 10720 10725 10730 10735 10740 10745 10750 10755 10760 10765 10770 10775 10780 10785 10790 10795 10800 10805 10810 10815 10820 10825 10830 10835 10840 10845 10850 10855 10860 10865 10870 10875 10880 10885 10890 10895 10900 10905 10910 10915 10920 10925 10930 10935 10940 10945 10950 10955 10960 10965 10970 10975 10980 10985 10990 10995 11000 11005 11010 11015 11020 11025 11030 11035 11040 11045 11050 11055 11060 11065 11070 11075 11080 11085 11090 11095 11100 11105 11110 11115 11120 11125 11130 11135 11140 11145 11150 11155 11160 11165 11170 11175 11180 11185 11190 11195 11200 11205 11210 11215 11220 11225 11230 11235 11240 11245 11250 11255 11260 11265 11270 11275 11280 11285 11290 11295 11300 11305 11310 11315 11320 11325 11330 11335 11340 11345 11350 11355 11360 11365 11370 11375 11380 11385 11390 11395 11400 11405 11410 11415 11420 11425 11430 11435 11440 11445 11450 11455 11460 11465 11470 11475 11480 11485 11490 11495 11500 11505 11510 11515 11520 11525 11530 11535 11540 11545 11550 11555 11560 11565 11570 11575 11580 11585 11590 11595 11600 11605 11610 11615 11620 11625 11630 11635 11640 11645 11650 11655 11660 11665 11670 11675 11680 11685 11690 11695 11700 11705 11710 11715 11720 11725 11730 11735 11740 11745 11750 11755 11760 11765 11770 11775 11780 11785 11790 11795 11800 11805 11810 11815 11820 11825 11830 11835 11840 11845 11850 11855 11860 11865 11870 11875 11880 11885 11890 11895 11900 11905 11910 11915 11920 11925 11930 11935 11940 11945 11950 11955 11960 11965 11970 11975 11980 11985 11990 11995 12000 12005 12010 12015 12020 12025 12030 12035 12040 12045 12050 12055 12060 12065 12070 12075 12080 12085 12090 12095 12100 12105 12110 12115 12120 12125 12130 12135 12140 12145 12150 12155 12160 12165 12170 12175 12180 12185 12190 12195 12200 12205 12210 12215 12220 12225 12230 12235 12240 12245 12250 12255 12260 12265 12270 12275 12280 12285 12290 12295 12300 12305 12310 12315 12320 12325 12330 12335 12340 12345 12350 12355 12360 12365 12370 12375 12380 12385 12390 12395 12400 12405 12410 12415 12420 12425 12430 12435 12440 12445 12450 12455 12460 12465 12470 12475 12480 12485 12490 12495 12500 12505 12510 12515 12520 12525 12530 12535 12540 12545 12550 12555 12560 12565 12570 12575 12580 12585 12590 12595 12600 12605 12610 12615 1262
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Gln₆₅ Leu Glu Arg Cys₇₀ Arg₇₀ Leu Gly Ala Glu Ser₇₅ Pro Ser Ile Ala Ser₈₀
 Thr Ser Ser Thr Glu₈₅ Lys Ser Phe Pro₉₀ Trp₉₀ Arg Ser Thr Asp Val₉₅ Pro
 Asn Thr Gly Val₁₀₀ Ser Lys Pro Arg Val₁₀₅ Ser Asp Ala Val Gln₁₁₀ Pro Asn
 Asn Tyr Leu₁₁₅ Ile Arg Thr Glu₁₂₀ Pro Glu Gln Gly Thr₁₂₅ Leu Tyr Ser Pro
 Glu Gln Thr Ser Leu His Glu₁₃₅ Ser Glu Gly Ser₁₄₀ Leu Gly Asn Ser Arg
 Ser₁₄₅ Ser Thr Gln Met₁₅₀ Asn Ser Tyr Ser Asp₁₅₅ Ser Gly Tyr Gln Glu Ala₁₆₀
 Gly Ser Phe His₁₆₅ Asn Ser Gln Asn Val₁₇₀ Ser Lys Ala Asp Asn Arg₁₇₅ Gln
 Gln His Ser Phe₁₈₀ Ile Gly Ser Thr Asn₁₈₅ Asn His Val Val₁₉₀ Arg Asn Ser
 Arg Ala Glu₁₉₅ Gly Gln Thr Leu Val₂₀₀ Gln Pro Ser Val₂₀₅ Ala Asn Arg Ala
 Met Arg₂₁₀ Arg Val Ser Ser Val₂₁₅ Pro Ser Arg Ala Gln₂₂₀ Ser Pro Ser Tyr
 Val₂₂₅ Ile Ser Thr Gly Val₂₃₀ Ser Pro Ser Arg Gly₂₃₅ Ser Leu Arg Thr Ser₂₄₀
 Leu Gly Ser Gly Phe₂₄₅ Gly Ser Pro Ser Val₂₅₀ Thr Asp Pro Arg Pro₂₅₅ Leu
 Asn Pro Ser Ala₂₆₀ Tyr Ser Ser Thr Thr₂₆₅ Leu Pro Ala Ala Arg₂₇₀ Ala Ala
 Ser Pro Tyr₂₇₅ Ser Gln Arg Pro Ala₂₈₀ Ser Pro Thr Ala Ile₂₈₅ Arg Arg Ile
 Gly Ser₂₉₀ Val Thr Ser Arg Gln₂₉₅ Thr Ser Asn Pro Asn₃₀₀ Gly Pro Thr Pro
 Gln Tyr Gln Thr Thr Ala₃₁₀ Arg Val Gly Ser Pro₃₁₅ Leu Thr Leu Thr Asp₃₂₀
 Ala Gln Thr Arg Val₃₂₅ Ala Ser Pro Ser Gln₃₃₀ Gly Gln Val Gly Ser₃₃₅ Ser
 Ser Pro Lys Arg₃₄₀ Ser Gly Met Thr Ala₃₄₅ Val Pro Gln His Leu₃₅₀ Gly Pro
 Ser Leu Gln Arg Thr Val His Asp₃₆₀ Met Glu Gln Phe Gly₃₆₅ Gln Gln Gln
 Tyr Asp₃₇₀ Ile Tyr Glu Arg Met₃₇₅ Val Pro Pro Arg Pro₃₈₀ Asp Ser Leu Thr
 Gly₃₈₅ Leu Arg Ser Ser Tyr₃₉₀ Ala Ser Gln His Ser₃₉₅ Gln Leu Gly Gln Asp₄₀₀
 Leu Arg Ser Ala Val₄₀₅ Ser Pro Asp Leu His₄₁₀ Ile Thr Pro Ile Tyr₄₁₅ Glu
 Gly Arg Thr Tyr₄₂₀ Tyr Ser Pro Val Tyr₄₂₅ Arg Ser Pro Asn His₄₃₀ Gly Thr
 Val Glu Leu₄₃₅ Gln Gly Ser Gln Thr₄₄₀ Ala Leu Tyr Arg Thr₄₄₅ Gly Val Ser
 Gly Ile₄₅₀ Gly Asn Leu Gln Arg₄₅₅ Thr Ser Ser Gln Arg₄₆₀ Ser Thr Leu Thr
 Tyr₄₆₅ Gln Arg Asn Asn Tyr₄₇₀ Ala Leu Asn Thr Thr₄₇₅ Ala Thr Tyr Ala Glu₄₈₀
 Pro Tyr Arg Pro Ile₄₈₅ Gln Tyr Arg Val Gln₄₉₀ Glu Cys Asn Tyr Asn₄₉₅ Arg
 Leu Gln His Ala₅₀₀ Val Pro Ala Asp Asp₅₀₅ Gly Thr Thr Arg Ser₅₁₀ Pro Ser
 Ile Asp Ser₅₁₅ Ile Gln Lys Asp Pro₅₂₀ Arg Glu Phe Ala Trp₅₂₅ Arg Asp Pro
 Glu Leu₅₃₀ Pro Glu Val Ile His₅₃₅ Met Leu Glu His Gln₅₄₀ Phe Pro Ser Val
 Gln Ala Asn Ala Ala₅₄₅ Ala Tyr Leu Gln His Leu₅₅₅ Cys Phe Gly Asp Asn₅₆₀
 Lys Val Lys Met Glu Val Cys Arg Leu Gly Gly Ile Lys His Leu Val

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565 570 575
 Asp Leu Leu Asp His Arg Val Leu Glu Val Gln Lys Asn Ala Cys Gly
 580 585 590
 Ala Leu Arg Asn Leu Val Phe Gly Lys Ser Thr Asp Glu Asn Lys Ile
 595 600 605
 Ala Met Lys Asn Val Gly Gly Ile Pro Ala Leu Leu Arg Leu Leu Arg
 610 615 620
 Lys Ser Ile Asp Ala Glu Val Arg Glu Leu Val Thr Gly Val Leu Trp
 625 630 635 640
 Asn Leu Ser Ser Cys Asp Ala Val Lys Met Thr Ile Ile Arg Asp Ala
 645 650 655
 Leu Ser Thr Leu Thr Asn Thr Val Ile Val Pro His Ser Gly Trp Asn
 660 665 670
 Asn Ser Ser Phe Asp Asp Asp His Lys Ile Lys Phe Gln Thr Ser Leu
 675 680 685
 Val Leu Arg Asn Thr Thr Gly Cys Leu Arg Asn Leu Thr Ser Ala Gly
 690 695 700
 Glu Glu Ala Arg Lys Gln Met Arg Ser Cys Glu Gly Leu Val Asp Ser
 705 710 715 720
 Leu Leu Tyr Val Ile His Thr Cys Val Asn Thr Ser Asp Tyr Asp Ser
 725 730 735
 Lys Thr Val Glu Asn Cys Val Cys Thr Leu Arg Asn Leu Ser Tyr Arg
 740 745 750
 Leu Glu Leu Glu Val Pro Gln Ala Arg Leu Leu Gly Leu Asn Glu Leu
 755 760 765
 Asp Asp Leu Leu Gly Lys Glu Ser Pro Ser Lys Asp Ser Glu Pro Ser
 770 775 780
 Cys Trp Gly Lys Lys Lys Lys Lys Lys Arg Thr Pro Gln Glu Asp
 785 790 795 800
 Gln Trp Asp Gly Val Gly Pro Ile Pro Gly Leu Ser Lys Ser Pro Lys
 805 810 815
 Gly Val Glu Met Leu Trp His Pro Ser Val Val Lys Pro Tyr Leu Thr
 820 825 830
 Leu Leu Ala Glu Ser Ser Asn Pro Ala Thr Leu Glu Gly Ser Ala Gly
 835 840 845
 Ser Leu Gln Asn Leu Ser Ala Ser Asn Trp Lys Phe Ala Ala Tyr Ile
 850 855 860
 Arg Gly Gly Arg Pro Lys Arg Lys Gly Leu Pro Ile Leu Val Glu Leu
 865 870 875 880
 Leu Arg Met Asp Asn Asp Arg Val Val Ser Ser Gly Ala Thr Ala Leu
 885 890 895
 Arg Asn Met Ala Leu Asp Val Arg Asn Lys Glu Leu Ile Gly Lys Tyr
 900 905 910
 Ala Met Arg Asp Leu Val Asn Arg Leu Pro Gly Gly Asn Gly Pro Ser
 915 920 925
 Val Leu Ser Asp Glu Thr Met Ala Ala Ile Cys Cys Ala Leu His Glu
 930 935 940
 Val Thr Ser Lys Asn Met Glu Asn Ala Lys Ala Leu Ala Asp Ser Gly
 945 950 955 960
 Gly Ile Glu Lys Leu Val Asn Ile Thr Lys Gly Arg Gly Asp Arg Ser
 965 970 975
 Ser Leu Lys Val Val Lys Ala Ala Ala Gln Val Leu Asn Thr Leu Trp
 980 985 990
 Gln Tyr Arg Asp Leu Arg Ser Ile Tyr Lys Lys Asp Gly Trp Asn Gln
 995 1000 1005
 Asn His Phe Ile Thr Pro Val Ser Thr Leu Glu Arg Asp Arg Phe Lys
 1010 1015 1020
 Ser His Pro Ser Leu Ser Thr Thr Asn Gln Gln Met Ser Pro Ile Ile
 1025 1030 1035 1040
 Gln Ser Val Gly Ser Thr Ser Ser Ser Pro Ala Leu Leu Gly Ile Arg
 1045 1050 1055
 Asp Pro Arg Ser Glu Tyr Asp Arg Thr Gln Pro Pro Met Gln Tyr Tyr
 1060 1065 1070

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Asn Ser Gln Gly Asp Ala Thr His Lys Gly Leu Tyr Pro Gly Ser Ser
 1075 1080 1085
 Lys Pro Ser Pro Ile Tyr Ile Ser Ser Tyr Ser Ser Pro Ala Arg Glu
 1090 1095 1100
 Gln Asn Arg Arg Leu Gln His Gln Gln Leu Tyr Tyr Ser Gln Asp Asp
 1105 1110 1115 1120
 Ser Asn Arg Lys Asn Phe Asp Ala Tyr Arg Leu Tyr Leu Gln Ser Pro
 1125 1130 1135
 His Ser Tyr Glu Asp Pro Tyr Phe Asp Asp Arg Val His Phe Pro Ala
 1140 1145 1150
 Ser Thr Asp Tyr Ser Thr Gln Tyr Gly Leu Lys Ser Thr Thr Asn Tyr
 1155 1160 1165
 Val Asp Phe Tyr Ser Thr Lys Arg Pro Ser Tyr Arg Ala Glu Gln Tyr
 1170 1175 1180
 Pro Gly Ser Pro Asp Ser Trp Val Tyr Asp Gln Asp Ala Gln Gln Arg
 1185 1190 1195 1200
 Asn Ser Phe Phe Leu Thr Leu Phe Arg Leu Arg
 1205 1210

(2) INFORMATION FOR SEQ ID NO:7:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 970 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

- (1x) FEATURE:
 (A) NAME/KEY: misc feature
 (B) LOCATION: 1..970
 (D) OTHER INFORMATION: /note= "Y2H9"

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GAATTCACAC	AGATAACACT	GCTGCTCCCG	CCCTTCGCT	CCTCGGCCGC	GCAATGGGCA	60
CCCGCGACGA	CGAGTACGAC	TACCTCTTTA	AAGTTGTCCT	TATTGGAGAT	TCTGGTGTG	120
GAAAGAGTAA	TCTCCTGTCT	CGATTTACTC	GAAATGAGTT	TAATCTGGAA	AGCAAGAGCA	180
CCATTGGAGT	AGAGTTTGCA	ACAAGAAGCA	TCCAGGTTGA	TGGAAAAACA	ATAAAGGCAC	240
AGATATGGGA	CACAGCAGGG	CAAGAGCGAT	ATCGAGCTAT	AACATCAGCA	TATTATCGTG	300
GAGCTGTAGG	TGCCTTATTG	GTTTATGACA	ITGCTAAACA	TCTACATAT	GAAAATGTAG	360
AGCGATGGCT	GAAAGAACTG	AGAGATCATG	CTGATAGTAA	CATTGTTATC	ATGCTTGTGG	420
GCAATAAGAG	TGATCTACGT	CATCTCAGGG	CAGTTCCTAC	AGATGAAGCA	AGAGCTTTTG	480
CAGAAAAGAA	TGGTTTGTC	TTCATTGAAA	CTTCGGCCCT	AGACTCTACA	AATGTAGAAG	540
CTGCTTTTCA	GACAATTTTA	ACAGAGATTT	ACCGCATTGT	TTCTCAGAAG	CAAATGTCAG	600
ACAGACGCGA	AAATGACATG	TCTCCAAGCA	ACAAATGTGGT	TCCTATTTCAT	GTTCCACCAA	660
CCACTGAAAA	CAAGCCAAAG	GTGCAGTGCT	GTCAGAACAT	CTAAGGCATT	TCTCTTCTCC	720
CCTAGAAGGC	TGTGTATAGT	CCATTTCCCA	GGTCTSASAT	TTAAATATAW	TTGTAATTCT	780
TGTGTCACTT	TTGTGTTTTA	TTACTTCATA	CTTATGAATT	TTTCCATGTC	CTAAGTCTTT	840
TGATTTTGMT	TTATAAAATC	ATCCACTTGT	NCCGAATGNC	TGCAGCTTTT	TTTCATGCTA	900
TGGCTTCACT	AGCCTTAGTT	TNATAAACTG	AATGTTTGGA	TTCTCCCCC	CAAAAAAAA	960
AAAACTCGAG						970

(2) INFORMATION FOR SEQ ID NO:8:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 264 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

- (1x) FEATURE:
 (A) NAME/KEY: misc feature
 (B) LOCATION: 1..264
 (D) OTHER INFORMATION: /note= "Y2H23b"

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(x1) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GAATTCGCGG CCGGTCGAC CCCCCACCCC CGATGCCACC ACCCCCANTS GGNTCTCCCN 60
 HCCGASTCAT CAGTTCTTCC ATGGNGTNCC CTGGTCTGRC CCTCCAGCT CCCCAGGCN 120
 TTCTCGGGGT CTGNCAGCAG CCNCCAGATT AACTCAACAG TGTACTCCC TGGGGGTGGG 180
 TCTGGNCCCC CTGANGATGT GAAGCCACCA GTCTNAGNGG TCCGGGGTCT GTACTGTCCA 240
 CCCCCTCCAG GTGGACCTGG CGCT 300

(2) INFORMATION FOR SEQ ID NO:9:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 340 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: misc feature
 (B) LOCATION: 1..340
 (D) OTHER INFORMATION: /note= "Y2H27"

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GAATTCGCGG CCGGTCGAC CCGGTCGCG TCGACCTGTT CCCCAGGCCC TAGAGGTCAT 60
 TCCTCGTACC CTGATCCAGA ACTGTGGGGC CAGCACCATC CGTCTACTTA CCTCCCTTCG 120
 GGCCAAGCAC ACCCAGGAGA ACTGTGAGAC CTGGGGTGTA AATGGTGAGA CGGGTACTTT 180
 GGTGGACATG AAGGAAGTGG GCATATGGGA GCCATTGGCT GTGAAGCTGC AGACTTATAA 240
 GACAGCAGTG GAGACGGCAG TTCTGCTACT GCGAATTGAT GACATCGTTT CAGGCCACAA 300
 AAAGAAAGGC GATGACCAGA GCCGGCAAGG CCGNGCTCCT 360

(2) INFORMATION FOR SEQ ID NO:10:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 404 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: misc feature
 (B) LOCATION: 1..404
 (D) OTHER INFORMATION: /note= "Y2H35"

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GAATTCGCGG TCGGTCGAC GGTTAGTCCC ACTGGNCGCA TCGAGGGHTT CACCAACGTC 60
 ATGGAGCTGT ATGGCANGAT CCGCGAGGTC TTCNCCTGC CAACTGCCGA GGTGATGTTT 120
 TGCACCCTGA NCACCCACAA AGTGGACATN GACAAGCTCC TGGGGGGCCA GATCGGGCTG 180
 GAGGACTTCA TCTTCGCCCC CGTGAAGGGG YAGCGCAAGG AGGTGGAGGT GTTCAWGTG 240
 GAGGATGYAC TCGGKCTCAC CATCACGGAC AACGGGGCTG GCTACGCTTC CATCAAGCGC 300
 ATCAAGGAGG GCAGCGTGAT CGACCACATC CACCTCATCA GCGTGGGCGA CATGATCGAG 360
 GCCATTAACG GGCAGAGCTT CCTGGGCTGC CGGCATTACG AGGT 420

(2) INFORMATION FOR SEQ ID NO:11:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 350 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: misc feature
 (B) LOCATION: 1..350
 (D) OTHER INFORMATION: /note= "Y2H171"

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GAATTCGCGG CCGGTCGAC AAAAAAAGTA AAAGGAAGTC GGCAAAATCTT ACCCCGCTG 60
 TTTACCAAAA ACATCACCTC TAGCATCACC AGTATTAGAG GCACCGCCTG CCCAGTGACA 120

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CATGTTTAAC GGCCGCGGTA CCTAACCGT GCRAAGGTAG CATAATCACT TGTTCCTTAA      180
GTAGGGACCT GTATGAATGG CTCCACGAGG GTTCAGCTGT CTCTTACTTT TAACCARTGA      240
AATTGACCTG CCGGTGAAGA GGCGGGCATG ACACAGCAAG ACGAGAAGAC CCTATGGAGC      300
TTTAATTTAT TAATGCAAAC AGTACCTAAC AAACCCACAG GTCCTTAAAC      350

```

(2) INFORMATION FOR SEQ ID NO:12:

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(1) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 350 base pairs
  (B) TYPE: nucleic acid
  (C) STRANDEDNESS: single
  (D) TOPOLOGY: linear

```

(ix) FEATURE:

```

  (A) NAME/KEY: misc feature
  (B) LOCATION: 1..350
  (D) OTHER INFORMATION: /note= "Y2H41"

```

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

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GAATTCGCGG NCGCGTCGAC AGATAATGAA AAAACCAGAG GTTCCCTTCT TTGGTCCCT      60
NNNNGATGGT GCTATTGTGA ATGGAAGGT TCTACCCATT ATGGTTAGAG CAACAGCTAT      120
AAATGCAAGC CGTGCTCTGA AATCTCTGAT TCCATTGTAT CAAAACCTCT ATGAGGAGAG      180
AGCACGATAC CTGCAAACAA TTGTCCAGCA CCACTTAGAA CCAACAACAT TTGAAGATTT      240
TGNAGCACAG GTTTTTTCTC CAGCTCCCTA CCACCATTTA CCATCTGATG CCSTTGGCTC      300
CTACCCAGAG ATTCTACCCA GTGAAAACCTC CCACAGCAAC GCAGGTAGGA      350

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CLAIMS

What is claimed is:

1. An isolated nucleic acid comprising a nucleotide sequence encoding at least a presenilin-interacting domain of a presenilin-interacting protein selected from the group consisting of a mammalian S5a (approximately residues 70-377 of SEQ ID NO: 2), GT24 (approximately residues 346-862 of SEQ ID NO: 4), p0071 (approximately residues 509-1022 of SEQ ID NO: 6), Rab11 (SEQ ID NO: 7),
5 retinoid X receptor- β (SEQ ID NO: 8), cytoplasmic chaperonin (SEQ ID NO: 9), Y2H35 (SEQ ID NO: 10), Y2H171 (SEQ ID NO: 11), and a Y2H41 (SEQ ID NO: 12) presenilin-interacting domain.
- 10 2. An isolated nucleic acid comprising a nucleotide sequence of at least 10 consecutive nucleotides selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, GenBank Accession Numbers F08730, T18858, X81889, X56740, X53143, M84820, X63522, M81766, U17104, X74801, R12984,
15 D55326, and T64843, and a sequence complementary to any of these sequences.
3. An isolated nucleic acid as in claim 2 comprising a nucleotide sequence of at least 15 consecutive nucleotides selected from said group.
- 20 4. An isolated nucleic acid as in claim 2 comprising a nucleotide sequence of at least 20 consecutive nucleotides selected from said group.

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5. An isolated nucleic acid comprising a nucleotide sequence encoding an antigenic determinant of a presenilin-interacting protein selected from the group consisting of a mammalian S5a, GT24, p0071, Rab11, retinoid X receptor- β , cytoplasmic chaperonin, Y2H35, Y2H171, and Y2H41 protein.
- 5
6. A method for identifying allelic variants or heterospecific homologues of a human presenilin-interacting protein gene comprising
- choosing a nucleic acid probe or primer capable of hybridizing to a human presenilin-interacting protein gene sequence under stringent hybridization conditions;
- 10 mixing said probe or primer with a sample of nucleic acids which may contain a nucleic acid corresponding to said variant or homologue;
- detecting hybridization of said probe or primer to said nucleic acid corresponding to said variant or homologue.
- 15 7. A method as in claim 6 wherein said sample comprises a sample of nucleic acids selected from the group consisting of human genomic DNA, human mRNA, and human cDNA.
8. A method as in claim 6 wherein said sample comprises a sample of nucleic
- 20 acids selected from the group consisting of mammalian genomic DNA, mammalian mRNA, and mammalian cDNA.
9. A method as in claim 6 wherein said sample comprises a sample of nucleic
- 25 acids selected from the group consisting of invertebrate genomic DNA, invertebrate mRNA, and invertebrate cDNA.

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10. A method as in claim 6 further comprising the step of isolating said nucleic acid corresponding to said variant or homologue.

5 11. A method as in claim 6 wherein said nucleic acid is identified by hybridization.

12. A method as in claim 6 wherein said nucleic acid is identified by PCR amplification.

10

13. A method for identifying allelic variants or heterospecific homologues of a human presenilin-interacting protein gene comprising

choosing an antibody capable of selectively binding to a human presenilin-interacting protein;

15 mixing said antibody with a sample of proteins which may contain a protein corresponding to said variant or homologue;

detecting binding of said antibody to said protein corresponding to said variant or homologue.

20 14. A method as in claim 13 wherein said sample comprises a sample of proteins selected from the group consisting of human proteins, human fusion proteins, and proteolytic fragments thereof.

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15. A method as in claim 13 wherein said sample comprises a sample of proteins selected from the group consisting of mammalian proteins, mammalian fusion proteins, and proteolytic fragments thereof.

5 16. A method as in claim 13 wherein said sample comprises a sample of proteins selected from the group consisting of invertebrate proteins, invertebrate fusion proteins, and proteolytic fragments thereof.

17. A method as in claim 13 further comprising the step of substantially
10 purifying said protein corresponding to said variant or homologue.

18. An isolated nucleic acid comprising an allelic variant or a heterospecific homologue of a human presenilin-interacting protein gene.

15 19. An isolated nucleic acid encoding an allelic variant or heterospecific homologue of a human presenilin-interacting protein.

20. An isolated nucleic acid comprising a recombinant vector including a nucleotide sequence of any one of claims 1-19.

20

21. An isolated nucleic acid as in claim 20 wherein said vector is an expression vector and said presenilin-interacting protein nucleotide sequence is operably joined to a regulatory region.

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22. An isolated nucleic acid as in claim 21 wherein said expression vector may express said presenilin-interacting protein sequence in mammalian cells.

5 23. An isolated nucleic acid as in claim 22 wherein said cells are selected from the group consisting of fibroblast, liver, kidney, spleen, bone marrow and neurological cells.

24. An isolated nucleic acid as in claim 21 wherein said vector is selected from
10 the group consisting of vaccinia virus, adenovirus, retrovirus, neurotropic viruses and Herpes simplex.

25. An isolated nucleic acid as in claim 21 wherein said expression vector
encodes at least a presenilin-interacting domain of a presenilin-interacting protein
15 selected from the group consisting of a mammalian S5a, GT24, p0071, Rab11,
retinoid X receptor- β , cytoplasmic chaperonin, Y2H35, Y2H171, and Y2H41 protein.

26. An isolated nucleic acid as in claim 21 wherein said vector further
comprises sequences encoding an exogenous protein operably joined to said
20 presenilin-interacting protein sequence and whereby said vector encodes a presenilin-
interacting protein fusion protein.

27. An isolated nucleic acid as in claim 26 wherein said exogenous protein is
selected from the group consisting of lacZ, trpE, maltose-binding protein, a poly-His

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tag, glutathione-S-transferase, a GAL4-DNA binding domain, and a GAL4 activation domain.

28. An isolated nucleic acid comprising a recombinant expression vector
5 including nucleotide sequences corresponding to an endogenous regulatory region of a presenilin-interacting protein gene.

29. An isolated nucleic acid as in claim 28 wherein said endogenous regulatory region is operably joined to a marker gene.

10

30. A host cell transformed with an expression vector of any one of claims 20-29, or a descendant thereof.

31. A host cell as in claim 30 wherein said host cell is selected from the group
15 consisting of bacterial cells and yeast cells.

32. A host cell as in claim 30 wherein said host cell is selected from the group consisting of fetal cells, embryonic stem cells, zygotes, gametes, and germ line cells.

20 33. A host cell as in claim 30 wherein said cell is selected from the group consisting of fibroblast, liver, kidney, spleen, bone marrow and neurological cells.

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34. A host cell as in claim 30 wherein said cell is an invertebrate cell.

35. A non-human animal model for Alzheimer's Disease, wherein a genome of said animal, or an ancestor thereof, has been modified by at least one recombinant
5 construct, and wherein said recombinant construct has introduced a modification selected from the group consisting of (1) insertion of nucleotide sequences encoding at least a functional domain of a heterospecific normal presenilin-interacting protein, (2) insertion of nucleotide sequences encoding at least a functional domain of a heterospecific mutant presenilin-interacting protein, (3) insertion of nucleotide
10 sequences encoding at least a functional domain of a conspecific homologue of a heterospecific mutant presenilin-interacting protein, and (4) inactivation of an endogenous presenilin-interacting protein gene.

36. An animal as in claim 35 wherein said modification is insertion of a
15 nucleotide sequence encoding at least a functional domain of a normal human presenilin-interacting protein selected from the group consisting of a mammalian S5a, GT24, p0071, Rab11, retinoid X receptor- β , cytoplasmic chaperonin, Y2H35, Y2H171, and Y2H41 protein.

20 37. An animal as in claim 35 wherein said modification is insertion of a nucleotide sequence encoding at least a functional domain of a mutant human presenilin-interacting protein selected from the group consisting of a mammalian S5a, GT24, p0071, Rab11, retinoid X receptor- β , cytoplasmic chaperonin, Y2H35, Y2H171, and Y2H41 protein.

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38. An animal as in claim 35 wherein said animal is selected from the group consisting of rats, mice, hamsters, guinea pigs, rabbits, dogs, cats, goats, sheep, pigs, and non-human primates.

5 39. An animal as in claim 35 wherein said animal is an invertebrate.

40. A method for producing at least a functional domain of a presenilin-interacting protein comprising culturing a host cell of any of claims 30-34 under suitable conditions to produce said presenilin by expressing said nucleic acid.

10

41. A substantially pure preparation of a protein selected from the group consisting of a mammalian S5a, GT24, p0071, Rab11, retinoid X receptor- β , cytoplasmic chaperonin, Y2H35, Y2H171, and Y2H41 protein.

15 42. A substantially pure preparation of a polypeptide comprising an amino acid sequence of at least 10 consecutive amino acid residues selected from the group consisting SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, and GenBank Accession Numbers F08730, T18858, X81889, X56740, X53143, M84820, X63522, M81766, U17104, X74801, R12984, D55326, and T64843.

20

43. A substantially pure preparation of a polypeptide as in claim 42 comprising an amino acid sequence of at least 15 consecutive amino acid residues selected from said group.

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44. A substantially pure preparation of a polypeptide comprising at least a presenilin-interacting domain of a presenilin-interacting protein selected from the group consisting of a mammalian S5a, GT24, p0071, Rab11, retinoid X receptor- β ,
5 cytoplasmic chaperonin, Y2H35, Y2H171, and Y2H41 protein.

45. A substantially pure preparation of a polypeptide comprising an antigenic determinant of a presenilin-interacting protein selected from the group consisting of a mammalian S5a, GT24, p0071, Rab11, retinoid X receptor- β , cytoplasmic
10 chaperonin, Y2H35, Y2H171, and Y2H41 protein.

46. A method of producing antibodies which selectively bind to a presenilin-interacting protein comprising the steps of
administering an immunogenically effective amount of a presenilin-
15 interacting protein immunogen to an animal;
allowing said animal to produce antibodies to said immunogen; and
obtaining said antibodies from said animal or from a cell culture derived therefrom.

20 47. A substantially pure preparation of an antibody which selectively binds to an antigenic determinant of a presenilin-interacting protein selected from the group consisting of a mammalian S5a, GT24, p0071, Rab11, retinoid X receptor- β , cytoplasmic chaperonin, Y2H35, Y2H171, and Y2H41 protein.

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48. A substantially pure preparation of an antibody as in claim 47 wherein said antibody selectively binds to an antigenic determinant of a mutant presenilin-interacting protein and fails to bind to a normal presenilin-interacting protein.

5 49. A cell line producing an antibody of any one of claims 47-48.

50. A method for identifying compounds which can modulate the expression of a presenilin-interacting protein gene comprising
contacting a cell with a test candidate wherein said cell includes a
10 regulatory region of a presenilin-interacting protein gene operably joined to a coding region; and
detecting a change in expression of said coding region.

51. A method as in claim 50 wherein said change comprises a change in a
15 level of an mRNA transcript encoded by said coding region.

52. A method as in claim 50 wherein said change comprises a change in a level of a protein encoded by said coding region.

20 53. A method as in claim 50 wherein said change is a result of an activity of a protein encoded by said coding region.

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54. A method as in claim 50 wherein said coding region encodes a marker protein selected from the group consisting of β -galactosidase, alkaline phosphatase, green fluorescent protein, and luciferase.

5 55. A method for identifying compounds which can selectively bind to a presenilin-interacting protein comprising the steps of
providing a preparation including at least one presenilin-interacting protein component;
contacting said preparation with a sample including at least one candidate
10 compound; and
detecting binding of said presenilin-interacting protein component to said candidate compound.

15 56. The method in 55 wherein said binding to said presenilin-interacting component is detected by an assay selected from the group consisting of: affinity chromatography, co-immunoprecipitation, a Biomolecular Interaction Assay, and a yeast two-hybrid system.

20 57. A method of identifying compounds which can modulate activity of a presenilin-interacting protein comprising the steps of
providing a cell expressing a normal or mutant presenilin-interacting protein gene;
contacting said cell with at least one candidate compound; and
detecting a change in a marker of said activity.

25

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58. A method as in claim 57 wherein measurement of said marker indicates a difference between cells bearing an expressed mutant presenilin-interacting protein gene and otherwise identical cells free of an expressed mutant presenilin-interacting protein gene.

5

59. A method as in claim 57 wherein said change comprises a change in a non-specific marker of cell physiology selected from the group consisting of pH; intracellular Ca^{++} , Na^+ , or K^+ ; cyclic AMP levels; GTP/GDP ratios; phosphatidylinositol activity; and protein phosphorylation.

10

60. A method as in claim 57 wherein said change comprises a change in expression of said presenilin-interacting protein.

61. A method as in claim 57 wherein said change comprises a change in intracellular concentration or flux of an ion selected from the group consisting of Ca^{++} , Na^+ and K^+ .

15

62. A method as in claim 57 wherein said change comprises a change in occurrence or rate of apoptosis or cell death.

20

63. A method as in claim 57 wherein said change comprises a change in production of $\text{A}\beta$ peptides.

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64. A method as in claim 57 wherein said change comprises a change in phosphorylation of at least one microtubule associated protein.

65. A method as in claim 57 wherein said cell is a cell cultured in vitro.

5

66. A method as in claim 65 wherein said cell is a transformed host cell of any one of claims 30-34.

67. A method as in claim 65 wherein said cell is explanted from a host bearing
10 at least one mutant presenilin-interacting protein gene.

68. A method as in claim 65 wherein said cell is explanted from a transgenic animal of any one of claims 35-39.

15 69. A method as in claim 57 wherein said cell is a cell in a live animal.

70. A method as in claim 69 wherein said cell is a cell of a transgenic animal of any one of claims 35-39.

20 71. A method as in claim 57 wherein said cell is in a human subject in a clinical trial.

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72. A diagnostic method for determining if a subject bears a mutant presenilin-interacting protein gene comprising the steps of

providing a biological sample of said subject;

5 detecting in said sample a mutant presenilin-interacting protein nucleic acid, a mutant presenilin-interacting protein, or a mutant presenilin-interacting protein activity.

73. A method as in claim 72, wherein a mutant presenilin-interacting protein
10 nucleic acid is detected by an assay selected from the group consisting of direct nucleotide sequencing, probe specific hybridization, restriction enzyme digest and mapping, PCR mapping, ligase-mediated PCR detection, RNase protection, electrophoretic mobility shift detection, and chemical mismatch cleavage.

15 74. A method as in claim 72, wherein a mutant presenilin-interacting protein is detected by an assay selected from the group consisting of an immunoassay, a protease assay, and an electrophoretic mobility assay.

75. A pharmaceutical preparation comprising a substantially pure presenilin-
20 interacting protein and a pharmaceutically acceptable carrier.

76. A pharmaceutical preparation comprising an expression vector operably
encoding a presenilin-interacting protein, wherein said expression vector may express
said presenilin-interacting protein in a human subject, and a pharmaceutically
25 acceptable carrier.

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77. A pharmaceutical preparation comprising an expression vector operably encoding a presenilin-interacting protein antisense sequence, wherein said expression vector may express said presenilin-interacting protein antisense sequence in a human
5 subject, and a pharmaceutically acceptable carrier.

78. A pharmaceutical preparation comprising a substantially pure antibody, wherein said antibody selectively binds to a mutant presenilin-interacting protein, and a pharmaceutically acceptable carrier.

10

79. A pharmaceutical preparation as in claim 78 wherein said preparation is essentially free of an antibody which selectively binds a normal presenilin-interacting protein.

15 80. A pharmaceutical preparation comprising a substantially pure preparation of an antigenic determinant of a mutant presenilin-interacting protein.

81. A pharmaceutical preparation as in claim 80 wherein said preparation is essentially free of an antigenic determinant of a normal presenilin-interacting protein.

20

82. A method of treatment for a patient bearing a mutant presenilin-interacting protein gene comprising the step of administering to said patient a therapeutically effective amount of the pharmaceutical preparation of any one of claims 75-81.

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83. A method as in claim 82, wherein said pharmaceutical preparation is targeted to a cell type is selected from the group consisting of heart, brain, lung, liver, skeletal muscle, kidney, pancreas and neurological cells.

5

INTERNATIONAL SEARCH REPORT

Int. Patent Application No.
PC/CA 97/00051

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C12N15/74 C12N5/10 C07K14/47 C07K16/18
A01K67/027 C12Q1/68 G01N33/577 A61K31/70 A61K38/17
A61K39/395 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K A01K C12N G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 35, 1 September 1995, pages 20615-20620, XP002029380 JOHANSSON, E., ET AL. ON: "MOLECULAR CLONING AND EXPRESSION OF A PITUITARY GLAND PROTEIN MODULATING INTESTINAL FLUID SECRETION" cited in the application see the whole document</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	<p>1-12, 18-20, 30,31, 34, 41-47,49</p>

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents:

- * "A" document defining the general state of the art which is not considered to be of particular relevance
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- * "O" document referring to an oral disclosure, use, exhibition or other means
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- * "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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- * "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- * "&" document member of the same patent family

Date of the actual completion of the international search

14 April 1997

Date of mailing of the international search report

25.04.97

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INTERNATIONAL SEARCH REPORT

International Application No.
PCT/CA 97/00051

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>EMBL SEQUENCE DATA LIBRARY, 20 December 1994, HEIDELBERG, GERMANY, XP002029381 PAWLAK, A., ET AL. : "CHARACTERIZATION OF A LARGE POPULATION OF mRNAs FROM HUMAN TESTIS" cited in the application ACCESSION No. T18858 see the whole document ---</p>	<p>1-12, 18-20, 30,31,34</p>
X	<p>EMBL SEQUENCE DATA LIBRARY, 17 February 1995, HEIDELBERG, GERMANY, XP002029382 AUFFRAY, C., ET AL. : "IMAGE: INTEGRATED MOLECULAR ANALYSIS OF THE HUMAN GENOME AND ITS EXPRESSION" cited in the application ACCESSION No. F08730 see the whole document ---</p>	<p>1-12, 18-20, 30,31, 34, 41-47,49</p>
X	<p>EMBL SEQUENCE DATA LIBRARY, 22 July 1994, HEIDELBERG, GERMANY, XP002029383 ZAHRAOUI, A., ET AL. : "CODING SEQUENCES OF HUMAN Rab8 AND Rab11 cDNAs" cited in the application ACCESSION No. X56740 see the whole document ---</p>	<p>1-12, 18-20, 30,31, 34, 41-47,49</p>
X	<p>EMBL SEQUENCE DATA LIBRARY, 19 February 1991, HEIDELBERG, GERMANY, XP002029384 DRIVAS, G.T., ET AL.: "IDENTIFICATION AND CHARACTERIZATION OF A HUMAN HOMOLOG OF THE SCHIZOSACCHAROMYCES POMBE RAS-LIKE GENE YPT-3" cited in the application ACCESSION No. X53143 see the whole document ---</p>	<p>1-12, 18-20, 30,31, 34, 41-47,49</p>
X	<p>EMBL SEQUENCE DATA LIBRARY, 7 February 1992, HEIDELBERG, GERMANY, XP002029385 CHAMBON, P., ET AL. : "PURIFICATION, CLONING, AND RXR IDENTITY OF THE HELA CELL FACTOR WITH WHICH RAR OR TR HETERODIMERIZES TO BIND TARGET SEQUENCES EFFICIENTLY" cited in the application ACCESSION No. M84820 see the whole document ---</p>	<p>1-12, 18-20, 30,31, 34, 41-47,49</p>

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INTERNATIONAL SEARCH REPORT

International Application No.

PC/CA 97/00051

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>EMBL SEQUENCE DATA LIBRARY, 31 March 1992, HEIDELBERG, GERMANY, XP002029386 FLEISCHHAUER, K., ET AL. : "ISOLATION OF A FULL-LENGTH cDNA CLONE ENCODING A N-TERMINALLY VARIANT FORM OF THE HUMAN RETINOID X RECEPTOR BETA" cited in the application ACCESSION No. X63522 see the whole document</p> <p style="text-align: center;">---</p>	<p>1-12, 18-20, 30,31, 34, 41-47,49</p>
X	<p>EMBL SEQUENCE DATA LIBRARY, 10 December 1991, HEIDELBERG, GERMANY, XP002029387 YU, V.C., ET AL. : "RXR-beta: A COREGULATOR THAT ENHANCES BINDING OF RETINOIC ACID, THYROID HORMONE, AND VITAMIN D RECEPTORS TO THEIR COGNATE RESPONSE ELEMENTS" cited in the application ACCESSION No. M81766 see the whole document</p> <p style="text-align: center;">---</p>	<p>1-12, 18-20, 30,31, 34, 41-47,49</p>
X	<p>EMBL SEQUENCE DATA LIBRARY, 7 January 1995, HEIDELBERG, GERMANY, XP002029388 SEVIGNY, G., ET AL. : "ASSIGNMENT OF THE HUMAN HOMOLOGUE OF THE mTRIC-P5 GENE (TRIC5) TO BAND 1q23 BY FLUORESCENCE IN SITU HYBRIDIZATION" cited in the application ACCESSION No. U17104 see the whole document</p> <p style="text-align: center;">---</p>	<p>1-12, 18-20, 30,31, 34, 41-47,49</p>
X	<p>EMBL SEQUENCE DATA LIBRARY, 1 January 1994, HEIDELBERG, GERMANY, XP002029389 WALKLEY, N.A., ET AL. : "CLONING, STRUCTURE AND mRNA EXPRESSION OF HUMAN Cctg, WHICH ENCODES THE CHAPERONIN SUBUNIT CCTgamma" cited in the application ACCESSION No. X74801 see the whole document</p> <p style="text-align: center;">---</p>	<p>1-12, 18-20, 30,31, 34, 41-47,49</p>
X	<p>EMBL SEQUENCE DATA LIBRARY, 22 April 1995, HEIDELBERG, GERMANY, XP002029390 HILLIER, L., ET AL. : "THE WashU-MERCK EST PROJECT" cited in the application ACCESSION No. R12984 see the whole document</p> <p style="text-align: center;">---</p>	<p>1-12, 18-20, 30,31, 34, 41-47,49</p>

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INTERNATIONAL SEARCH REPORT

International Application No.

PCT/CA 97/00051

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBL SEQUENCE DATA LIBRARY, 25 August 1995, HEIDELBERG, GERMANY, XP002029391 FUJIWARA, T., ET AL.: "UNPUBLISHED" cited in the application ACCESSION No. D55326 see the whole document ---	1-12, 18-20, 30,31,34
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